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Investigation of mammary blood flow changes by transrectal colour Doppler sonography in an *Escherichia coli* mastitis model

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51.

To my parents

FELIX QUI POTUIT RERUM COGNOSCERE CAUSAS

PUBLIUS VERGILIUS MARO
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Index of abbreviations

[α]	Doppler-angle (°)
a.i.	after infusion
B-mode	brightness modulation
BE	bacteriological examination
[BFV]	blood flow volume (l/min)
[c]	velocity of sound in tissue (1540 m/s)
CFU	colony-forming unit
CMT	California Mastitis Test
[D]	end-diastolic frequency shift (Hz)
EC	electrical conductivity
E. coli	*Escherichia coli*
[f]	Doppler-shift frequency (Hz)
[fo]	transmitted frequency (Hz)
GH	growth hormone
IGF	insulin-like growth factor
[Min]	minimal diastolic frequency shift (Hz)
MY	milk yield
p.i.	post infectionem
PGE	prostaglandin E
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
[PI]	Pulsatility Index
PMN	polymorphonuclear neutrophilic leukocytes
p.p.	post partum
[RI]	Resistance Index
ROS	reactive oxygen species
RT	rectal temperature
[S]	maximal systolic frequency shift (Hz)
SCC	somatic cell count
[TAMF]	time averaged maximum frequency shift (Hz)
TCDS	transrectal colour Doppler sonography
[v]	blood flow velocity (m/s)

1. Introduction

Bovine mastitis is still ranked among the main production diseases in dairy herds of developed countries (Miller et al. 1993; Rajala-Schultz & Gröhn 1999; Bradley et al. 2007). Approximately, every third cow in Germany suffers from mastitis once per lactation and a financial impact of about € 0.02 per one litre milk produced is estimated (Fehlings 2008). In 2007 nearly 15% of all animal losses in Germany were based on udder diseases, such as mastitis (ADR 2007).

Especially, intramammary infections with *Escherichia coli (E. coli)* very often lead to an acute mastitis with severe clinical effects (Bannermann et al. 2004). Occuring mainly in the peripartum and the early stage of lactation applied therapies are in many cases insufficient or futile (Olde Riekerink et al. 2008). The consequence of failed therapy can be the total loss of individual animals or enormous economic losses during current and / or subsequent lactations (Seegers et al. 2003). A single case of a total animal loss caused by *E. coli* mastitis leads to an estimated loss of at least € 1800 (costs in case of successful therapy: about € 180).

The identification of the inflammatory event at an early stage is not only a basic demand of milk hygiene and an essential precondition for a successful therapy (Grunert 1990), but also an important factor for the limitation of mammary tissue damages (Burvenich et al. 2003) and economic impacts due to yield losses (De Mol & Ouweltjes 2001).

Clinical examination, California Mastitis Test (CMT) (Schalm & Noorlander 1957) and bacteriological examinations (BE) are the most common non-invasive methods for diagnosing mastitis. Beyond that, electrical conductivity (EC) has been investigated for years but is still not sufficiently precise for an early detection of an acute mastitis (Hovinen et al. 2006).

New methods such as measuring volatile components and potentiometric values of milk (Eriksson et al. 2005; Mottram et al. 2007), as well as a new biosensor system that analyses lactose and EC (Culina et al. 2006) or infrared thermography (Hovinen et al. 2008; Colak et al. 2008) are still under development. The need for a non-invasive early detection method not being related to the act of milking (advantage: before first calving, dry period, clinical signs appear before milk changes) is obvious.

From studies with invasive blood flow measurements a close relationship between inflammatory processes of the mammary gland and mammary blood flow rate is known (Dhondt et al. 1977a). Effects of metabolic activities of the mammary gland on mammary blood flow seem to be evident (Lough et al. 1990).

In this present study a new non-invasive and quantitative method was used for examining the pudendoepigastric trunk by transrectal colour Doppler sonography (TCDS). The investigated vessel section is the only pre-section of the inguinally running external pudic artery, the major supplier of the mammary gland of cows, which is transrectally accessible (Bragulla & König 1999; Budras & Wünsche 2002). Former studies have shown that TCDS is an established non-invasive method for detecting physiological (Bollwein et al. 2002; Krueger et al. 2009) or pathological (Schmauder 2003; Rauch et al. 2008) changes of genital organs in cattle.

Thus, this study had two goals: to establish TCDS as a non-invasive and precise method for evaluating the physiological blood flow volume (BFV) of the pudendoepigastric trunk in a homogeneous group of cows with a well defined udder health status; and, also, to use this technique for investigating pathological mammary blood flow changes at an early stage of udder inflammation using a stringent *E. coli* mastitis model.

2. Physiology of the mammary blood flow in cows

2.1 Vascularization of the mammary gland

Arterial system: The bovine mammary gland is mainly supplied by the external pudic artery emanating from the abdominal aorta and branching off the external iliac artery and the femoral artery. Additionally, also the internal pudic artery participates in supplying the udder. Pre-located to the external pudic artery a short pre-inguinal vessel section, the pudendoepigastric trunk, is found. After passing the inguinal canal and approaching the udder basis sigmoidally (s-shaped flexure), it divides into the anterior and posterior mammary artery. The s-shaped flexure allows for downward distension of the udder as it fills with milk, without stressing the blood vessel. While the anterior mammary artery is responsible for supplying the corresponding cranial and caudal udder quarters including their teats, the posterior mammary artery essentially supplies the rear udder quarter. These vessels go on branching when descending down into the gland. In literature an artery is described leading off the branching angle of the external pudic artery. Numerous variations of the runs and the vessels leading off are possible. Only small amounts of blood reach the mammary gland by the perineal artery (from the internal iliac artery) supplying the upper rear portion of the udder. Basically, there is no cross over of blood supply between the left and the right udder half influencing milk production (Budras & Wünsche 2002).

Venous system: Homonymous veins leave the mammary gland anti-parallel to the arteries and open out into the external and internal pudic vein. The subcutaneous abdominal vein exits the udder at the anterior end of the front quarters and passes along the abdominal wall. This large vein being visible under the belly skin of the cow enters the body cavity at the xiphoid process via milk wells. Carrying less than 10% of blood leaving the udder the perineal vein leaves the rear of the mammary gland anti-parallel to the perineal artery (Budras & Wünsche 2002).

2.2 Regulation of mammary blood flow

To date, regulation of mammary blood flow is still only poorly understood. It is not clear whether mammary metabolism regulates mammary blood flow, or mammary blood flow regulates mammary metabolism.

Continuous blood flow is an essential precondition for the physiological function of the lactating mammary gland in cows. Thereby, the system of blood supply is developed proportionately to the intensity of metabolism (Huth 1995a). By slowing down the blood flow velocity in the udder the uptake of nutrients is facilitated. This happens by an increase of the venous capacity of the factor 50 compared to the arteries (Gravert 1983).

In fact, there is a variety of agents influencing the regulation of mammary blood flow, but only little is known about their exact mechanisms. In the following, an itemization of substances with proven effects on mammary blood flow is given.

Above all, hormones stimulating the growth of the udder parenchyma also stimulate the development of mammary vessels (*metabolic hormones:* growth hormones (GH), corticosteroids, thyroid hormones, insulin, gastrointestinal hormones such as somatostatin, gastrin, cholecystokinin; *reproductive hormones:* oestrogen, progesterone, placental lactogen, prolactin, oxytocin; *growth factors:* insulin-like growth factor (IGF)-I, epidermal growth factor, transforming growth factor-α, transforming growth factor-β). Some of these hormones are engaged in the regulation of mammary blood flow, too (Svennersten-Sjaunja & Olsson 2005).

Mammary blood vessels are affected by several vasoactive hormones such as adrenaline, noradrenaline, angiotensin II and vasopressin (vasoconstrictors) or nitric oxide and atrial natriuretic peptide (vasodilators) (Cvek 1997).

It is described that direct intravenous infusions of adrenaline in sheep lead to decreases of mammary blood flow of 50% compared to initial values (Leenanuruksa & McDowell 1985). Especially, during stressful situations the release of adrenaline and noradrenaline

causes a sudden and marked reduction in mammary blood flow, because of vasoconstriction. From goats, it is known that moments of enhanced stress can lower udder blood flow to half and recovery to initial values takes 30 to 60 minutes (Linzell & Rasmussen 1972). This phenomenon should also be kept in mind, when carrying out measurements of mammary blood flow in cows. Particularly, effects of "stressful situations" like milking or oestrus should be examined.
Intravenous injections of vasopressin and oxytocin lead to an increase of mammary blood flow (Dhondt et al. 1973).

In principle, as a basic demand of correct Doppler sonographic measurements providing unbiased results, any unusual excitement of the examined animals must be avoided. Therefore, an appropriate adaption phase of the animals onto the unknown observation procedure, the examining persons and the environment is essential.

Also prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) are described as sex hormones increasing mammary blood flow. $PGF_{2\alpha}$ showes a higher influence on mammary blood flow than PGE_2 does (Dhondt et al. 1977b). This information should also be considered and verified, when evaluating mammary blood flow at different stages of the oestrus cycle.

A transection of mammary nerves of lactating cows (Peeters et al. 1949) and sheep (Peeters et al. 1952) leads to a distinct mammary vasodilation, indicating that the vascular tone is normally maintained in part by the mammary sympathetic innervation. Nevertheless, mammary vasculature is also regulated by a variety of locally produced, vasoactive agents. These substances act on the arterioles, capillaries, or both by changing vascular resistance and exchange function in the mammary gland (Prosser et al. 1996).
Additionally, in goats a decrease of mammary blood flow has been detected in denervated udders upon starvation (Chaiyabutr et al. 1980). This fact also implies that vasoconstriction is affected by local or humoral factors and is not only a response of the sympathetic nervous system (Davis & Collier 1985).

In goats, it has been shown that infusions of IGF-I and IGF-II close to the external pudic artery are able to increase mammary blood flow by acting directly on mammary tissues (Prosser et al. 1990; Prosser et al. 1994).

Experimental treatments with thyroxine and GH cause an increase of 35% and 33%, respectively, of mammary blood flow in cows (Davis et al. 1988). However, there is no evidence of GH having a direct effect on mammary tissue (McDowell & Hart 1984), and indeed there is some evidence indicating the contrary (Bauman & McCutcheon 1985).

The intensity of blood supply is closely related to milk production (Davis & Collier 1985). Basically, mammary blood flow of nonlactating cows is much lower than in lactating ones (Kensinger et al. 1983). This close correlation between bovine mammary blood flow per unit weight of tissue and milk yield is shown in the table reproduced below (Linzell & Rasmussen 1972):

Milk yield	Blood flow
dry	23 ml/min/100g mammary tissue
very low	26 ml/min/100g mammary tissue
high	87 ml/min/100g mammary tissue

The mammary blood flow increases in proportion of the cardiac output (Linzell 1974). An increased cardiac output, influenced by an intensified heart action and an increased heart volume, is a basic mechanism to handle enhanced demands of blood supply during lactation (Gravert 1983).

For example, the mammary gland of a Jersey cow, with 350 kg body weight, at peak lactation is perfused by 15.6% (7 l/min) of cardiac output (45 l/min) when producing 16 kg of milk daily (Davis et al. 1983). Thus, approximately every four minutes the equivalent of the total blood volume is received by the mammary gland. As a rule of

thumb, an amount of 540 l blood perfusing the udder is necessary for producing one litre milk (Huth 1995b).

With onset of lactation, increases of total (body) blood volume, cardiac output, mammary blood flow and blood flow through the gastrointestinal tract and liver occur. These changes are aimed at the supplying of the mammary gland with nutrients and hormones for the regulation of milk synthesis (Svennersten-Sjaunja & Olsson 2005). A decrease of mammary blood flow leads to an entire reduction of mammary nutrient uptake (Delamaire & Guinard-Flament 2006).

Another study has shown, that mammary BFV rapidly increases on the first day post partum (p.p.). Interestingly, BFV starts decreasing from day 1 to day 7 p.p., while MY goes on increasing during this time. A slight increase of BFV is reported for the high lactation period from day 14 to day 84 p.p. In literature both a tremendous decrease of BFV between the 21st and 33rd week of pregnancy and an increase of BFV between the 36th and 39th week of pregnancy are described. Although the intensity of blood supply is closely related to milk production, there are different degrees of correlation between mammary BFV and MY depending on the stage of lactation. In early lactation (day 7 to 56) mammary BFV is lowly correlated with MY, whereas in late lactation (21st to 29th week of pregnancy) BFV is moderately related to MY (Honnens et al. 2007).

From goats it is known, that an increase of intramammary pressure going along with an accumulation of milk seems not to affect mammary blood flow, at least in the short term, although the rate of milk secretion is decreased. However, a reduction of blood flow is seen when intramammary pressure increases severely to approximately twice the physiological maximum (Peaker 1980).

From lactating goats it is also known that intramammary infusions of colchicine lead to a distinct decrease of MY, but to a maintenance or even an increase of mammary blood flow (Henderson & Peaker 1980) associated with pyrexia and a local inflammatory response (Burvenich & Peeters 1980).

Also, changes of mammary blood flow caused by inflammatory events related to acute mastitides of cows are known. After an intramammary contact with a pathogen like *E. coli*, processes regulated by biochemical mediators provoke a dilatation of the arterioles, capillaries and venules. Increased hydrostatic pressure affects transudation and hyperaemia in the concerned tissue (Meurer 1999). An increased volume of blood is moved into the mammary region. In a former study measuring bovine mammary blood flow by an implanted electromagnetic flow probe after intramammary infusion of *E. coli* endotoxin, two peaks of mammary blood flow were found (Dhondt et al. 1977a). A first peak occurred at the third hour after infusion (a.i.) followed by a temporary return to the initial level and a second peak between the tenth and eleventh hour a.i. followed by a return to control levels between the thirteenth and fourteenth hour a.i.. Particular attention should be turned to this phenomenon, when examining mammary blood flow by other studies and methods.

3. Bovine Mastitis

3.1 Definition and Classification

Bovine mastitis is characterised by an inflammation of the whole mammary gland. Milk producing areas as well as milk storing and revulsive areas are affected. It is almost solely caused by invasion of bacteria. However, also viral, algal and fungal-related mastitides are known (Pyörälä 2003).

A classification into sub-clinical, clinical (mild, moderate, severe) and chronic forms can be made depending on the severity of inflammation (Hamann & Fehlings 2002):

Sub-clinial mastitides are characterised by an inflammation of the udder without any clinical signs. Somatic cell counts (SCC) are increased and pathogens are detectable.

An occurring of milk flakes in the foremilk without any visible or palpable alteration of the udder is the characteristic of a *mild clinical mastitis*.

In *moderate to severe clinical mastitides*, the affected animals show clearly clinical alterations of the udder (temperature, swelling, pain) and of the milk appearance. Pyrexia is prevalent.

The duration of the illness is described by the terms acute, chronic and subacute. Thereby, an *acute mastitis* is characterised by a rapid onset and a short course. In contrast, a *chronic mastitis* is characterised by a non-healing illness of long duration leading to atrophic or abnormal udder quarters in the end. The condition between acute and chronic is defined as *subacute*.

Thereby, the nature of the causative pathogen as well as the age, breed (Brolund 1985; Elbers et al. 1998), lactation state and number (Zadoks et al. 2001) as well as the immunological status of the animal play an important role (Viguier et al. 2009).

3.2 *Escherichia coli* mastitis

In most cases intrammamary infections with *E. coli* induce acute or peracute, serofibrinous mastitides with severe clinical symptoms, such as pyrexia, rapid respiration, decreased rumen motility, diarrhoea, amyostasia and inappetence. Increased firmness, heat and painful swelling of the udder can be observed as local signs. Watery milk with clots or flakes is the consequence of massive exudation with necrosis of the mammary tissue. Rapid progression of the disease leads to decreased milk production or to the total loss of milk yield within hours. In very severe courses hypersalivation, a very high pulse rate, hypothermia, paralysis or even death are not unusual (Burvenich et al. 2003).

E. coli is an acidogenic, lactose-fermenting, Gram negative bacterium, that is classified as part of the *Enterobacteriaceae* family of *gamma proteobacteria* (Hahn et al. 2005). This rod shaped pathogen is a facultative anaerobic and opportunistic mastitis causing pathogen (Barrow & Hill 1989; Nemeth et al. 1994), that is ubiquitous in cows´ environment (Burvenich et al. 2003). Unlike the enteropathic and bacteriaemic strains represented by a low number of *E. coli* serotypes (China & Goffaux 1999), the pathogens isolated from clinical bovine mastitides belong to a wide range of serological groups.
Entering the teat canal and proliferating in the lumen of the mammary gland is the most common path of intramammary *E. coli* infections (galactogeneous infection). Also haematogenous and percutaneous infections of the udder are possible (Acland 1995). Thereby, lesions caused by parturition can release pathogens from the gut, which move into the udder. Identical *E. coli* serotypes can be found in mastitic milk and in faeces (Burvenich et al. 2003).

After establishment of *E. coli* in the udder, the endotoxin lipopolysaccharide is released (De Schepper et al. 2008). Pathogen modulated cytokine production with a pro-inflammatory immune reaction is the consequence. Attracted by cytokines, specifically polymorphonuclear neutrophilic leukocytes (PMN) invade the mammary tissue by diapedesis. PMN migration through the mammary tissue leads to a damage of the milk producing epithelium and thereby to a continuous decline of MY (Nourshargh et al. 1992). After entering the cisterns, ducts and alveolar lumen of the mammary gland, PMN

phagocytose bacteria and eliminate them by production of reactive oxygen species (ROS) and enzymes. Excessive ROS production causes mammary tissue damage. The filling up of alveoli with harmful exudate as a result of the inflammatory cascade is apparently the most plausible explanation of mammary tissue damages during *E. coli* mastitis.

Neither adhesion at the gland´s epithelium nor special virulence factors are necessary for manifestation of an intramammary *E. coli* infection (Opdebeeck et al. 1988). The ability to proliferate in the milk secretion under nearly anaerobic conditions by metabolising lactose is widely sufficient (Hogan et al. 1992). A rapid and uncontrolled growth of *E. coli* with a huge number of bacteria in the udder cistern is not unusual, especially, related to the time of parturition and early lactation (Vandeputte-Van Messom et al. 1993; Shuster et al. 1996). Moreover, *E. coli* is also able to persist in the udder during the dry period (Bradley & Green 2001). It is assumed that more than half of all *E. coli* mastitides occurring within the first two months of lactation are related to infections during the dry period (Smith et al. 1985).

E. coli is supposed to be the most common cause of severe mastitis. A considerable increase of incidences of *E. coli* mastitides since 1960 is undoubted (Menzies et al. 1995). Particularly, around parturition and during the early lactation of so called “high producing cows” the incidence of *E. coli* mastitides with grave systemic clinical symptoms is eminent (Hill 1991). An extreme sensitivity for intramammary infections with ordinary environmental pathogens like coliforms is the reason for this phenomenon because of the reduced capability of the bovine immune system to fight pathogens during these periods (Heynemann et al. 1990; Kremer et al. 1993). Thereby, the sensitivity for coliform mastitis is influenced by several “cow factors”, such as age of the animal, stage of the lactation, leaking milk between milkings (Schukken et al. 1991; O´Reilly et al. 2006), increased teat-end roughness (severe hyperkeratosis) (Breen et al. 2009), cow cleanliness (Ward et al. 2002), genetic resistance, SCC, nutritional status, periparturient diseases (Peeler et al. 1994) or vaccination status (Paape et al. 2002).
During the lactation period the risk of infection is mainly prevalent in the times between milkings. In mid and late lactation the incidence of *E. coli* mastitides is decreasing (Huszenicza et al. 2004).

It is not the serotype of the bacterium, but the level of bacterial count that is the essential determinant for the severity of affection (Hogan & Smith 2003; Wenz et al. 2006). The level of bacterial counts in milk and the loss of milk production, especially, of the non-infected udder quarters, are directly related to the severity of *E. coli* mastitis (Vandeputte-Van Messom et al. 1993; Dosogne et al. 1997). However, the severity of *E. coli* mastitis is also more strongly influenced by "cow factors", such as the periparturient period, parity (Vangroenweghe et al. 2004a,b), the early stage of lactation, metabolic and hormonal alterations and a negative energy balance rather than by the pathogen itself (Burvenich et al. 2003). A decreased severity of *E. coli* mastitis and an increased self-curing rate is observed after peak lactation (Burvenich et al. 2003).

Dairy herds with a low level of SCC on average exhibit a higher incidence of environmental mastitis compared to dairy herds with a high level of SCC (Green et al. 1996; Suriyasathaporn et al. 2000). Older cows are more often affected and show higher numbers of colony forming units (CFU) (Van Werven et al. 1997; Pyörälä SHK & Pyörälä EO 1998). Both average SCC level and mastitis incidence are higher in older cows. Intramammary infections with *E. coli* always go along with negative correlations between increasing SCC and decreasing milk yields (Raubertas & Shook 1982; Deluyker 1991). The reason for this is damage to great parts of the mammary epithelium caused by invading bacteria and / or phagocytes.

Decreased or lost MY is a very serious aspect of clinical mastitis. The total lactational output that would have been expected might never be achieved again by once "high producing cows" that have suffered from severe acute mastitis. For the rest of the current lactation a permanent attenuated milk production may occur. Thereby, mastitides developed before the cow´s peak of lactation preponderate extremely (Burvenich et al. 2003).

4. Mammary blood flow measurements by Doppler sonography

In the past, several methods for measuring mammary blood flow of ruminants were used. The first direct measurement of mammary blood flow was made applying continuous thermodilution technique (Linzell 1957). Beside the antipyrine absorption method (Rasmussen 1965), the nitrous oxide diffusion (Reynolds et al. 1968) and the electromagnetic induction (Dhondt et al. 1977a; Kensinger et al. 1983), surgically implanted ultrasonic flow probes (Thivierge et al. 2000; Delamaire & Guinard-Flament 2006) were used.

Another tool for examining mammary blood flow in ruminants is the technology of Doppler sonography, even though, only a few superficial studies have been made in goats (Christensen et al 1989; Nielsen et al. 1990) and cows (Piccione et al. 2004a; Braun & Hoegger 2008) on this topic so far.

Doppler sonography is a non-invasive method for examining blood flow rates and velocities in blood vessels and tissues. It is based on the correspondent effect specified by the German natural scientist Johann Christian Doppler in 1842.

4.1 Basic principles of Doppler sonography

Ultrasonic waves emitted by oscillated piezoelectric crystals and impinging on body tissues of different densities are partly reflected at tissue interfaces (Waite et al. 1990; Flückiger 1997). Hitting moving structures, such as membranes of flowing erythrocytes, leads to a reflection with frequency shift, commonly known as Doppler-shift frequency (Dudwiesus et al. 1993; Marsál 1993). Depending on the transducer´s transmission frequency, the angle of intercept between the ultrasound beam and blood flow (Doppler angle / angle of insonation), the ultrasound velocity in the tissue (in soft biologic tissue: 1540 m/s) and the velocity of the reflector (e.g. moving red blood cells), Doppler-shift frequency is calculated in the following manner:

$$[f] = \frac{2 \times (fo \times v \times \cos\alpha)}{c}$$

[f] = Doppler-shift frequency (Hz)

[fo] = transmitted frequency (Hz)

[v] = blood flow velocity (m/s)

[α] = Doppler angle (°)

[c] = velocity of sound in tissue (1540 m/s)

In colour Doppler sonography frequency shift is colour-coded in a window and projected across the usual B-mode (brightness modulation of the dots or pixels on the echo-display screen) picture. Normally, blood stream moving towards the ultrasound probe is displayed in red colour, whereas blood stream moving away from the ultrasound probe is displayed in blue colour. By means of the colour intensity inferences on the frequency shift level can be made. The brighter the colour appears, the higher the frequency shift level is (Deane 1995).

In a coordinate system Doppler-shift frequency can be plotted against time as a spectral curve (Fig. I). Blood flow moving towards the transducer face is illustrated above the base line, whereas blood flow moving away from the transducer face is illustrated below the base line. For arterial vessels characteristic Doppler waves with different blood flow velocities during systole and diastole are the result (Dudwiesus et al. 1993).

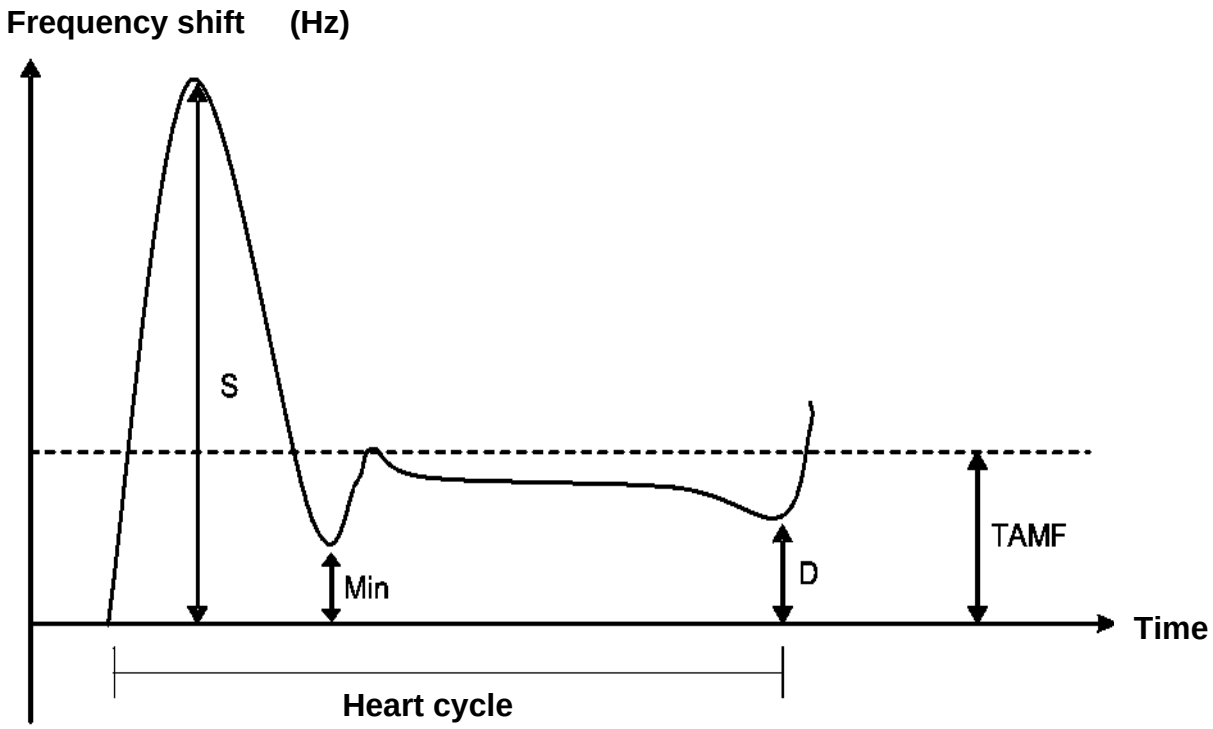

Fig. I: Schematic illustration of a Doppler wave with the maximal systolic (S), the minimal diastolic (Min), the end-diastolic (D) and the time averaged maximum (TAMF) frequency shift during one heart cycle (based upon Bollwein et al. 2000).

4.2 Instrumental technology

Today, mainly pulsed-wave-Doppler units are used. Piezoelectric crystals working alternatingly as transmitter and receiver are characteristic of these units. Thereby, only signals returning after a certain time interval are recorded. Unlike continuous-wave-Doppler units their advantage of depth-selective blood flow measurement is eminent by setting exactly the measurement depth and gearing the measurement window towards the vessel section of interest (Dudwiesus et al. 1993; Dickey 1997).

Simultaneous displaying of Doppler waves and B-mode pictures in the real-time mode (Duplex-mode) is another helpful advantage for an accurate localisation of the measurement area (Pensel & Warnking 1993).

In the Triplex-mode the B-mode picture is overlayed with a colour window which can be varied in size and position.

4.3 Methods of analysis

Measurements carried out by colour Doppler sonography can be analysed qualitatively, semi-quantitatively and quantitatively.

Qualitative analysis

The method of qualitative analysis is defined as a descriptive assessment of Doppler waves. Thereby, attention is payed to the existence of a diastolic blood flow, its direction and continuity during one heart cycle (Goswamy & Steptoe 1988; Tekay et al. 1996). However, blood flow evaluation is relatively subjective and difficult to standardize using this method of analysis (Dudwiesus et al. 1993).

Semi-quantitative analysis

For semi-quantitative analyses Doppler angle-independent indices are used, allowing an illustration of the blood flow resistance prevailing in the periphery of examined vessel sections. These indices are calculated from S, Min, D, and TAMF of one heart cycle. The higher the index value turns out, the higher the blood flow resistance of the organ supplied by the investigated blood vessel is (Dickey 1997). [S/D-ratio] (Stuart et al. 1980), Resistance Index [RI] (Pourcelot 1974) and Pulsatility Index [PI] (Gosling & King 1975; Dickey 1997) are the most common indices.

$$[\text{S/D ratio}] = \frac{S}{D}$$

$$[\text{RI}] = \frac{S - D}{S}$$

$$[\text{PI}] = \frac{S - Min}{TAMF}$$

Quantitative analysis

Quantitative analyses of Doppler sonographic measurements are carried out by evaluating blood flow velocity and blood flow volume. For evaluating the blood flow volume the angle of intersection between the ultrasound beam and blood flow (Doppler angle) as well as the diameter of the blood vessel must be known. By transposing the Doppler equation blood flow velocity is calculated.

$$[v] = \frac{f \times c}{2 \times fo \times \cos\alpha}$$

$$[Q] = v \times A$$

[Q] = blood flow volume (l/min)

[A] = diameter of the blood vessel (m)

Only measurements at a Doppler angle of under 60° provide significant Doppler waves. Little inaccuracies with an angle of over 60° produce relatively big errors in calculating the cosine and, thus, the blood flow velocity (Deane 1995). Ideally the angle of insonation is always kept between 20 and 50° (personal communication Poulsen Nautrup).

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Investigation of mammary blood flow changes by transrectal colour Doppler sonography in an *Escherichia coli* mastitis model

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The objectives of this preliminary investigation were to evaluate the feasibility of transrectal colour Doppler sonography (TCDS) for determining blood flow of the pudendoepigastric trunk in cows with experimentally induced *Escherichia coli* Mastitis. Five primiparous Holstein dairy cows, 4–6 months after calving, were examined in two trials. All monitored udder quarters were initially clinically healthy, somatic cell count (SCC) was <50 000 cells/ml and bacteriologically negative. The blood flow of the left and the right pudendoepigastric trunk was described by the blood flow volume (BFV). In the methodological part of the study, the intra-observer precision of the method was evaluated. The coefficients of variation of the BFV were 7·1% for the left and 9·4% for the right pudendoepigastric trunk. The intraclass correlation coefficients of the BFV were 0·99 ($P<0·001$) for the left and 0·75 ($P=0·004$) for the right vessel. BFV did not differ significantly between the left and the right side nor between pre- and post-milking nor between oestrus and dioestrus. In the experimental part of the study, significant differences of increasing BFV between 0 and 12 h p.i. (post infectionem) ($P=0·043$) and decreasing BFV between 12 and 24 h p.i. ($P=0·043$) were discovered for the pudendoepigastric trunk of the infected right side. In the left-right (control-infection) comparison a significant increase of the right BFV was observed at 12 h p.i. ($P=0·043$). The difference of an increasing SCC correlated positively with the difference of an increasing BFV between 0 and 12 h p.i. (Spearman's $\rho=1·00$; $P=0·043$) for the right infected side. It was shown that TCDS is a reproducible technique for investigating pathological mammary blood flow changes at an early stage of acute mastitis.

Keywords: Doppler sonography, mammary blood flow, pudendoepigastric trunk, *Escherichia coli* mastitis, cow.

Mastitis is still ranked among the main production diseases in dairy herds of developed countries (Miller et al. 1993; Rajala-Schultz & Gröhn, 1999). Especially intramammary infections with *Escherichia coli* very often lead to an acute mastitis with severe clinical effects (Bannermann et al. 2004). Enormous economic losses during current and/or subsequent lactations are consequences (Seegers et al. 2003). The identification of the inflammatory event at an early stage is not only a basic demand of milk hygiene and an essential precondition for a successful therapy (Grunert, 1990) but also an important factor for the limitation of economic impacts due to yield losses (De Mol & Ouweltjes, 2001). Effects of metabolic activities of the mammary gland on mammary blood flow seem to be evident (Lough et al. 1990). Clinical examination, California mastitis test (CMT) and bacteriological examinations (BE) are the most common non-invasive methods for diagnosing mastitis. In this present study a new non-invasive and quantitative method was used for examining the pudendoepigastric trunk by transrectal colour Doppler sonography (TCDS). The investigated vessel section is the only section of the inguinally running external pudic artery, the major supplier of the mammary gland of cows, which is transrectally accessible (Budras & Wünsche, 2002). Earlier studies have shown that TCDS is an established non-invasive method for detecting physiological (Bollwein et al. 2002; Krueger et al. 2009) or pathological (Rauch et al. 2008) changes of genital organs in cattle. The objectives of this preliminary investigation were to evaluate the feasibility of TCDS for determining blood flow of the pudendoepigastric

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Table 1. Clinical scores of five cows (A, B, C, D, E) using three parameters to assess the clinical status in the experimental part of the study. Individual scores and the sum of the scores of each animal at different times are shown. Also shown is the Median (Minimum/Maximum) per time of these sums. (NAD = Nothing Abnormal Detected)

			0 h					12 h					24 h				
Parameter	Score	Assessment	Animal					Animal					Animal				
			A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
	0	*NAD*	0	0	0	0	0										
	1	*mild impairment*						1	1	1	1		1				
Milk appearance	2	*moderate impairment*										2					
	3	*severe impairment*												3	3	3	3
	0	*NAD*	0	0	0	0	0										
	1	*mild impairment*															
Udder consistency	2	*moderate impairment*							2	2	2	2					
	3	*severe impairment*						3					3	3	3	3	3
	0	*NAD*	0	0	0	0	0										
	1	*mild impairment*								1		1					
General condition	2	*moderate impairment*						2	2		2		2	2			
	3	*severe impairment*													3	3	2
	0	*NAD*	0	0	0	0	0										
	1 to 3	*mild impairment*															
Sum	4 to 6	*moderate impairment*						6	5	4	5	5	6				
	7 to 9	*severe impairment*												8	9	9	8
		Median (min/max)			0					5 (4/6)					8 (6/9)		

trunk in cows with experimentally induced *Esch. coli* mastitis.

Materials and Methods

Animals

The study included a homogeneous group of five primiparous Holstein dairy cows, 120–180 days in milk, not pregnant and with an average milk yield (MY) of 28·6±3·6 kg/d. None of the animals had ever shown any signs of mastitis before. Weekly regular milk tests during a 3-week adaptation phase made by staff at the Clinic for Ruminants and the Milchprüfring Bayern e.V. (Registered Association of Milk Quality Control in Bavaria) guaranteed all udder quarters were clinically healthy, somatic cell count (SCC) was <50 000 cells/ml and milk samples were bacteriologically negative at the beginning of the trial. Milk was always sampled before regular milking. Bacteriological examinations were performed from foremilk samples; SCC was determined in 10 ml whole milk after each udder quarter had been milked separately with a quarter milker (WestfaliaSurge, Bönen, Germany).

During the adaptation phase rectal temperatures (RT), MY and CMT were documented twice a day. Also, the clinical status of the animals was defined twice daily by a clinical score (CS) with a change-scale from 0 (Nothing Abnormal Detected) over 1 (mild impairment) and 2 (moderate impairment) to 3 (severe impairment) quantifying milk appearance, udder consistency after palpation and general condition of the animals (Table 1). During the investigation phase, consisting of the methodological and the experimental parts of the study, all of these parameters (BE, RT, MY, CMT, SCC and CS) were documented at the times of measurements (establishment of the method: oestrus/dioestrus; *Esch. coli* mastitis model: 0/12/24 h). During the adaptation phase as well as during the investigation phase all animals were kept under constant conditions at the Clinic for Ruminants, Ludwig-Maximilians-University Munich, Germany. The trial was conducted under the approval and supervision of the Ethics Committee of the regional government in Munich, Germany (No 55.2-1-54-2531-108-05).

Stage of the oestrus cycle

For comparable hormonal conditions, all cows were synchronized by two injections of (+)-cloprostenol (Dalmazin®, Selectavet, Weyarn, Germany) at 12-d intervals. For an accurate determination of oestrus and dioestrus, palpations and ultrasound scanning of the ovaries as well as clinic-established and cattle-adapted laboratory techniques for detecting sexual steroids were used (Prakash et al. 1987).

Scanning procedure

All TCDS measurements were carried out using an ultrasound unit (Hitachi EUB 8500, Hitachi Medical Systems GmbH, Wiesbaden, Germany) equipped with a 7·5 MHz linear rectal transducer (Hitachi EUP-033J; Hitachi Medical Systems GmbH). The range of the Doppler gate

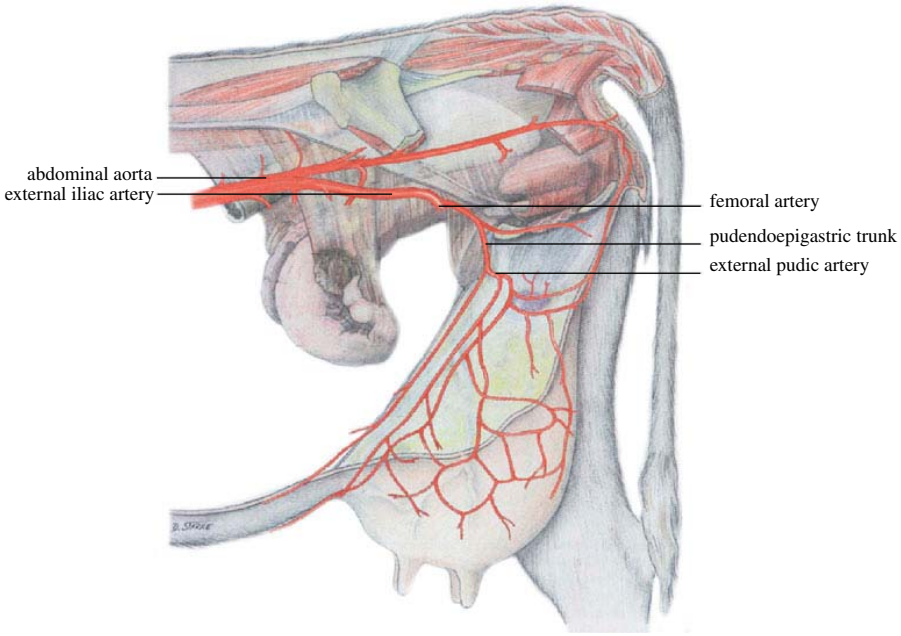

Fig. 1. Anatomical location of the pudendoepigastric trunk in cows (based upon Budras & Wünsche 2002).

was adapted to the diameter of the pudendoepigastric trunk. Measurements were shown on-line at the display together with the ultrasonic image. The 'frozen' B-mode/moving Doppler signals were saved on hard disk and later recorded on DVD for analysis. The transverse section of the blood vessel was recorded in the B-mode as a 'frozen' image. All evaluations were made afterwards using a specific ultrasound image viewer program (US Image Viewer, PCS-UV 1; 2002, 2004; Hitachi Medical Corporation).

The blood vessels of interest were found transrectally in the following manner (Fig. 1). By holding the face of the ultrasound transducer dorsally it was easy to identify the abdominal aorta. The external iliac artery was found at the level of the branching of the abdominal aorta. By following the external iliac artery and passing the femoral artery the pudendoepigastric trunk was located cranioventral of the cranial edge of the osseous pelvis.

Measurements were always performed by the same person in the same quiet room to which the animals had been conditioned during their adaptation phase. To study mammary blood flow, the left and the right pudendoepigastric trunk were examined. After correction for the specific angle at which the measurements had been taken, all waveforms of the blood flow were obtained at an interrogation angle between the Doppler ultrasound beam and the flow direction from 20 to 50 degrees. Both the left and the right pudendoepigastric trunk were scanned to determine the blood flow expressed by the blood flow volume (BFV, l/min). To calculate the BFV the area of the pudendoepigastric trunk was sized three times (Bollwein et al. 2004) by circumscribing the vessel internally with the cursor of the PC and then taking the average of all measurements.

Establishment of the method (*methodological part of the study*)

Precision of TCDS was verified in the clinically healthy animals. For that purpose all scanning was done before feeding time. Measurements were carried out within 2 h before and after milking during oestrus and dioestrus; thus, four measurements were taken per animal. For the intraobserver precision three separate measurements on each side were taken with an interval of 1–10 min. From each of the three measurements two consecutive Doppler waveforms with identical systolic and end-diastolic amplitudes were analysed and averaged (Bollwein et al. 1998; Bollwein et al. 2000; Bollwein et al. 2004). Pre- and post-milking recordings of the left and the right pudendoepigastric trunk were made during oestrus as well as during dioestrus.

Escherichia coli *mastitis model* (*experimental part of the study*)

Three days after the second injection of (+)-cloprostenol the animals were inoculated with pathogens during oestrus. Twenty international units of oxytocin (Veyx-Pharma, Schwarzenborn, Germany) were applied intravenously and the udder was stripped completely. The teats were

cleaned and disinfected with 70% ethanol and 500 CFU *Esch. coli* strain 1303 were administered intracisternally in the right rear quarter through the teat canal. Two millilitres of 0·9% sterile pyrogen-free saline without bacteria was inoculated into the left rear quarter as placebo (Petzl et al. 2008). Following administration of *Esch. coli* 1303 to a single udder quarter, all inoculated animals suffered from acute mastitis. Major clinical symptoms, such as udder swelling, milk appearance and general condition, were typical for an acute *Esch. coli* mastitis (Table 1). All milk samples from the inoculated quarters were positive for *Esch. coli* bacteria, whereas neighbouring quarters remained bacteriologically negative during the observation period. Measurements by TCDS of the left and the right pudendoepigastric trunk were performed during this experimental part at time 0 (immediately before inoculation), 12 and 24 h post infectionem (p.i.), always before milking.

Statistical methods

Statistical analyses were performed using the statistics program NCSS (NCSS, PASS, & GESS, Kaysville, USA, www.ncss.com), SPSS (version 15.0, SPSS, Chicago, USA, www.spss.com), SAS (version 9.1, SAS, Cary, USA, www.sas.com) and Excel (Microsoft R Office Excel 2003 (11.8033.8036) SP2).

Establishment of the method (*methodological part of the study*)*:* Ranges, means, standard errors and differences were calculated for all data measured on the left and the right pudendoepigastric trunk, before and after milking, during oestrus and dioestrus. Intra-observer precision, meaning reproducibility, was determined by the coefficient of variation (CV) and the intraclass correlation coefficient (ICC) in accordance with previous studies (Bollwein et al. 2000; Hollis et al. 2001). Differences between left and right, between pre- and post-milking, and between oestrus and dioestrus were compared using the non-parametric Wilcoxon signed-rank test (WSRT) owing to the limited number of animals and the non-normally distributed data (Bortz, 1993). Additionally, a repeated measures model (RMM) was calculated with the three individual values to confirm the results of the WSRT (SAS, ProcMixed). Results of $P<0·05$ were considered significant.

Esch. coli *mastitis model* (*experimental part of the study*)*:* For comparing the courses of BFV, RT, MY and SCC medians and quartiles were displayed. RMM was applied to examine the courses of these parameters. In addition, for comparisons between two different time points, the WSRT was applied again. Correlations between BFV and MY/SCC were evaluated using Spearman's rank correlation coefficient.

Table 2. Intra-observer precision and left-right comparison of the blood flow volume (BFV; l/min) of the left and the right pudendoepigastric trunk of five cows (A, B, C, D, E) in the methodological part. Values describe ranges and means and SE of three separate measurements on each side. Intra-observer precision is expressed by the coefficient of variation (CV), which is presented for each side of each individual cow (no significant difference in the precision between left and right, $P=0·345$)

Animal	Side	Range (l/min)	Mean ± SE (l/min)	CV (%)
A	*Left*	9·3–9·7	9·5 ± 0·1	2·2
	Right	6·0–7·2	6·5 ± 0·4	10·2
B	*Left*	5·2–6·2	5·6 ± 0·3	10·1
	Right	6·1–7·3	6·7 ± 0·4	9·0
C	*Left*	9·3–10·5	9·7 ± 0·4	6·4
	Right	7·7–8·0	7·9 ± 0·1	2·0
D	*Left*	4·7–5·5	5·1 ± 0·2	7·5
	Right	7·2–8·6	7·9 ± 0·4	9·2
E	*Left*	3·7–4·5	4·1 ± 0·2	9·5
	Right	4·2–5·8	4·8 ± 0·5	16·8
Mean	*Left*	3·7–10·5	6·8 ± 1·2	7·1
Mean	*Right*	4·2–8·6	6·8 ± 0·6	9·4
Overall	Mean	3·7–10·5	6·8 ± 1·3	8·3

Results

Establishment of the method (*methodological part of the study*)

Intra-observer precision and the left-right comparison of the BFV of the left and the right pudendoepigastric trunk of five cows is shown in Table 2. The CV of the BFV was 7·1% (6·8 ± 2·6 l/min) for the left and 9·4% (6·8 ± 1·3 l/min) for the right pudendoepigastric trunk, an average of 8·3% (6·8 ± 1·9 l/min). The ICC of the BFV was 0·99 ($P<0·001$) for the left and 0·75 ($P=0·004$) for the right vessel. The BFV of the left side did not differ significantly from the BFV of the right side (WSRT: $P=0·893$; RMM: $P=0·410$). The pre-post-milking comparison and the oestrus-dioestrus comparison are illustrated in Table 3. Values of BFV, means of left and means of right measurements did not differ significantly between pre- and post-milking (WSRT: left $P=0·345$; right $P=0·345$; RMM: left $P=0·141$; right $P=0·536$) or between oestrus and dioestrus (WSRT: left $P=0·690$; right $P=0·893$; RMM: left $P=0·646$; right $P=0·829$).

Esch. coli *mastitis model* (*experimental part of the study*)

The BFV differed significantly in the course of the trial between the left and the right side (RMM: $P<0·001$). Significant differences of increasing BFV between 0 and 12 h p.i. (WSRT: $P=0·043$) and decreasing BFV between 12 and 24 h p.i. (WSRT $P=0·043$) were discovered for the pudendoepigastric trunk of the infected right side (Fig. 2A). No significant differences were

Table 3. Pre-post-milking comparison and oestrus-dioestrus comparison of the blood flow volume (BFV; l/min) of the left and the right pudendoepigastric trunk of five cows in the methodological part of the study. Values describe ranges and means and SE of measurements of all animals on each side and differences between pre- and post-milking as well as between oestrus and dioestrus (measured only pre-milking)

Side	Time	Range (l/min)	Mean ± SE (l/min)	Difference ± SE (l/min)	Time	Range (l/min)	Mean ± SE (l/min)	Difference ± SE (l/min)
Left	*Pre-milking*	4·2–9·7	6·8 ± 1·2	0·7 ± 1·3	*Oestrus*	4·0–9·6	6·6 ± 1·0	0·2 ± 1·3
Left	*Post-milking*	4·6–8·2	6·1 ± 0·7		*Dioestrus*	4·2–9·7	6·8 ± 1·2	
Right	*Pre-milking*	4·8–7·9	6·8 ± 0·6	0·2 ± 0·9	*Oestrus*	5·0–9·9	6·9 ± 0·9	0·1 ± 1·5
Right	*Post-milking*	5·9–7·6	6·6 ± 0·3		*Dioestrus*	4·8–7·9	6·8 ± 0·6	
Mean	*Pre-milking*	4·2–9·7	6·8 ± 1·4	0·5 ± 1·1	*Oestrus*	4·0–9·9	6·8 ± 1·4	0·0 ± 1·3
Mean	*Post-milking*	4·6–8·2	6·3 ± 0·8		*Dioestrus*	4·2–9·7	6·8 ± 1·4	

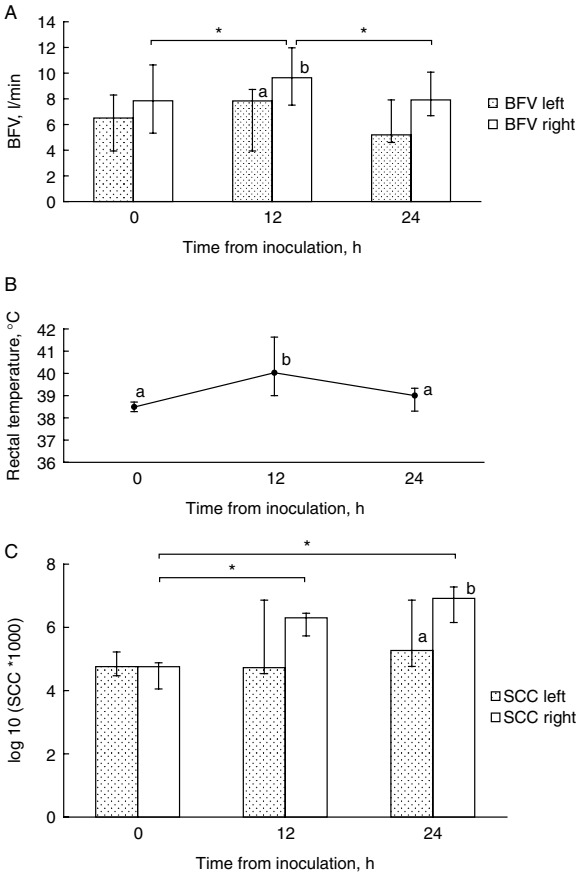

Fig. 2. 24-h course of (A) the blood flow volume [BFV; l/min], (B) the rectal temperature [RT; °C] and (C) the somatic cell count [$\log_{10}$ (SCC*1000)] of five cows in the experimental part, expressed by medians and quartiles (first and third quartiles indicated by error bars) during an induced *Escherichia coli* mastitis. Different letters indicate a statistically significant difference (A, C) between the left and the right side or (B) between different times ($P<0·05$), * indicates a statistically significant difference (A, C) between different times ($P<0·05$).

detected for the BFV of the left non-infected side ($P>0·05$). In the left-right comparison a significant difference of BFV was observed at the time of 12 h p.i. ($P=0·043$).

A significant time effect in the rectal temperature was seen (RMM: $P=0·006$), whereby a significant increase of the RT could be detected between 0 and 12 h p.i. (WSRT: $P=0·043$; RMM: $P=0·011$) as well as a statistically significant decrease between 12 and 24 h p.i. (WSRT: $P=0·043$; RMM: $P=0·037$) (Fig. 2B).

Between 12 and 24 h p.i. a significant decrease of MY was seen in the infected right udder half ($P=0·042$) (Fig. 3). Between 0 and 24 h p.i. both the left and right udder halves showed significant differences (WSRT: left $P=0·043$; right $P=0·043$; RMM: left $P=0·007$; right $P=0·026$). The left-right comparison showed no significant difference. Between 0 and 12 h p.i. the difference in the MY, which was decreasing, was correlated with the difference in the BFV of the right pudendoepigastric trunk, which was increasing ($\rho=0·60$; $P=0·043$).

In the RMM of the log 10 (SCC*1000) a significant time effect ($P<0·001$), side effect ($P<0·001$) and a significant time-side interaction effect ($P=0·004$) was found. SCC of the right udder quarters increased significantly between 0 and 12 h p.i. (WSRT: $P=0·043$; RMM: $P<0·001$) (Fig. 2C). Between 0 and 24 h p.i. the right udder half showed a significant increase in SCC (WSRT: $P=0·043$; RMM: $P<0·001$). A significant difference of the SCC was observed in the left-right comparison at the time of 24 h p.i. (WSRT: $P=0·043$; RMM: $P=0·004$). The correlation between BFV and SCC was examined by comparing the differences of the absolute values between different times. The difference of an increasing SCC correlated positively with the difference of an increasing BFV between 0 and 12 h p.i. ($\rho=1·00$; $P=0·043$) for the right side. Between 12 and 24 h p.i. the difference of an increasing SCC correlated negatively with the difference of a decreasing BFV ($\rho=-0·60$; $P=0·043$) for the right pudendoepigastric trunk.

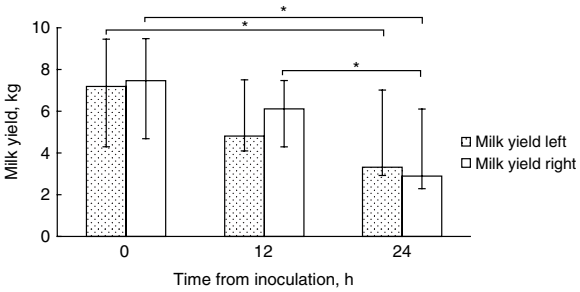

Fig. 3. 24-h course of the milk yield (kg; average of the two left quarters *v.* average of the two right quarters) of five cows in the experimental part, expressed by medians and quartiles (first and third quartiles indicated by error bars) during an induced *Escherichia coli* mastitis. * indicates a statistically significant difference between different times ($P<0{\cdot}05$).

Discussion

Establishment of the method (*methodological part of the study*)

In the beginning of this trial the intra-observer precision of this new method was necessarily assessed and possible dependences on disturbance variables (left/right; pre-/post-milking; oestrus/dioestrus) were tested. In the literature several methods of evaluating intra-observer precision are described (Lamb et al. 1999; Bollwein et al. 2000; Schmucker et al. 2000). In the present study measurements were taken three times on each side and precision was expressed by the CV and the ICC similarly to other studies (Bollwein et al. 2000; Hollis et al. 2001). Intra-observer precision of repeatedly performed measurements in single animals was consistently good. The overall mean, using left and right measurements of all five animals, also supported this finding with a CV of 8·3%. It was demonstrated that TCDS of the pudendoepigastric trunk provides well reproducible and, thus, precise values. The method was precise, as it repeatedly measured the same values. However, a precise method does not necessarily mean a correct method. It is conceivable that the method measures precisely values which are a certain amount higher or lower than the real value. To evaluate the correctness of the method further investigations would be needed to compare TCDS with other methods, such as direct blood measurements. On the basis of former studies using Doppler sonography for different investigations, bilateral comparisons were drawn for information about relevancies and differences of the two scanning locations, the left and the right pudendoepigastric trunk (Christensen et al. 1989; Bollwein et al. 1998; Schmucker et al. 2000; Bollwein et al. 2002; Piccione et al. 2004a). We did not find a significant difference between measurements of the left and the right pudendoepigastric trunk; however, the animal number in the present study was limited. Yet, a power analysis using a SD of 0·5, which was found in the present study, calculated that a difference in the BFV of at least 1 l/min between left and right would have been identified with a power of 90% and alpha=0·05 using the five animals (calculated with NCSS/PASS). This indicates similar blood support of both udder halves and equates with almost equally distributed MY of the left and the right udder halves (data not shown) when kept under physiological conditions as in this trial. The results also corroborate findings of other authors using Doppler ultrasound for measurements of mammary blood flow velocities at different udder-associated vessels in lactating cows (Piccione et al. 2004a), goats (Christensen et al. 1989) and ewes (Piccione et al. 2004b).

A significant difference between measurements before and after milking could not be discovered. Similar BFV observations are reported for mammary blood flow measurements in lactating goats (Christensen et al. 1989). Earlier studies using invasive methods at the external pudic artery in cows showed that positive changes of BFV are only measurable during milking. An abrupt and enormous increase of mammary blood flow with onset of milking followed by a very quick return of BFV within minutes to initial values at the end of milking has been described (Houvenaghel et al. 1973; Davis & Collier, 1985). This can be seen as an explanation for not detecting any differences between pre- and post-milking measurements within 2 h in the present trial.

In goats and cattle it is known that the oestrus cycle affects blood flow in certain blood vessels. In goats the mammary BFV is decreased the day before and on the day of oestrus, but no alteration is seen on any other days during the oestrus cycle (Burvenich, 1980). In the present trial a significant difference of BFV between scannings during oestrus and dioestrus was not detected.

Esch. coli *mastitis model* (*experimental part of the study*)

Since observations of the early stage of an acute mastitis are not possible under field conditions a standardized and, thus, very well reproducible stringent *Esch. coli* mastitis model was used for this trial.

After pathogen inoculation a significant difference of BFV in the right infected udder half between 0 und 12 h p.i. was detected. The first change of BFV can be easily explained by the basic principles of inflammation. After contact with the pathogen, processes regulated by biochemical mediators provoke a dilatation of the arterioles, capillaries and venules. Increased hydrostatic pressure affects transudation and hyperaemia (Meurer, 1999). An increased volume of blood is moved into the mammary region. In an earlier study, measuring mammary blood flow (absolute integration values/unit of time; %) by an implanted electromagnetic flow probe after intramammary infusion of *Esch. coli* endotoxin two blood flow peaks were found (Dhondt et al. 1977). A first peak occurred at the third hour after infusion followed by a temporary return to the initial level and a second peak between the tenth and eleventh hour followed by a return to control

levels between the thirteenth and fourteenth hour. Since, in our study, measurements were made at 0, 12 and 24 h p.i. detection of the described first blood flow peak was not possible. However, the described second peak could be entirely validated by TCDS.

Significant differences of simultaneously increasing RT and SCC in the infected quarter between 0 and 12 h p.i. also fit well with the inflammatory procress with systemic and cellular defence mechanisms during the acute phase. Thereby, the statistically positive correlation between the changes of the right SCC and the changes of the right BFV clarifies well the degree of acute inflammation and its effect on mammary blood flow. A significant difference of BFV between the right (infected) and the left (non-infected) side at 12 h p.i. is remarkable because it allows the exact identification of the infected udder half.

Although the decrease of the MY of the right infected udder half was not statistically significant, the change of the MY correlated positively with the change of the increasing right BFV. At this stage of experimental *Esch. coli* mastitis it is known that mammary tissue shuts down synthesis of milk constituents and favours the expression of antimicrobial effector molecules such as defensins (Vanselow et al. 2006, Petzl et al. 2008). This could explain the increase of the BFV up to 12 h p.i. due to proinflammatory actions, although MY is already decreasing. Between 12 and 24 h p.i. the BFV of the right pudendoepigastric trunk (infected side) decreased significantly accompanied by significantly decreasing RT and MY (right). These findings also follow the basic inflammatory principles: hours after the occurrence of inflammation the dilatation of arterioles and the arterial branch still continues, whereas the venous branch and small veins start to constrict. Hyperaemia and a slowdown of blood flow are the consequences (Meurer, 1999). A lower volume of blood is moved into the mammary region. After the acute phase of inflammation RT returns to a lower level. The significant decrease of MY of the right infected side can be explained by the ongoing progression of the systemic illness which influences, typically for *Esch. coli* mastitis, the MY of the left non-infected side (Burvenich et al. 1999). A high correlation between decreasing MY of the uninfected gland and bacterial growth of the infected gland is known (Dosogne et al. 1997).

With this present study it could not only be shown that TCDS is a new non-invasive and precise method for evaluating the BFV of the pudendoepigastric trunk in healthy cows, but also that this technique was successfully used for detecting pathological mammary blood flow changes at an early stage in a stringent *Esch. coli* mastitis model. The discoveries of this study mark an important basis for further investigations of mammary blood flow, especially for experimental set-ups of infection models with udder pathogens and the associated detection of clinical, acute/chronic or subclinical mastitis by TCDS. Findings of this study provide significant information for bilateral comparisons and scanning times in mastitis infection models and should be seen as a helpful guideline for planned Doppler sonographic testings. It has to be noted that only initially udder-healthy animals with an equally distributed MY in both udder halves were examined in the present trial. Animals with pre-existing impairments of the udder and such with unequal milk distribution in the udder halves could have biased the results of the standardized study. This fact underlines the particular meaning and suitability of the findings described here with regard to well-defined mastitis infection models. Setting a 'zero-standard', meaning healthy-, left/right- and hour 0-status, of the single test animal before trial initiation is an absolute prerequisite for any examination.

Especially with a view to the severe problem of mastitis appearing worldwide and the need to detect it as early as possible, it is conceivable that TCDS may find its way from an experimental technique to a clinical application as another useful non-invasive and reliable tool for diagnosing pathological changes in the mammary gland of cows. However, more studies including some on the economical and logistical practicability of the method are needed.

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References

Bannermann DD, Paape MJ, Lee JW, Zhao X, Hope JC & Rainard P 2004 *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clinical and Diagnostic Laboratory Immunology* **11** 463–472

Bollwein H, Maierl J, Mayer R & Stolla R 1998 Transrectal color Doppler sonography of the A. uterina in cyclic mares. *Theriogenology* **49** 1483–1488

Bollwein H, Meyer HHD, Maierl J, Weber F, Baumgartner U & Stolla R 2000 Transrectal Doppler sonography of uterine blood flow in cows during the estrous cycle. *Theriogenology* **53** 1541–1552

Bollwein H, Baumgartner U & Stolla R 2002 Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. *Theriogenology* **57** 2053–2061

Bollwein H, Weber F, Woschée I & Stolla R 2004 Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. *Theriogenology* **6** 499–509

Bortz J 1993 [Chapter 5: Verification methods of difference hypotheses]. In: *Statistik für Sozialwissenschaftler 4th Edn*, pp. 144–145 Berlin, Germany: Springer Verlag

Budras KD, Wünsche A 2002 [Arteries, veins and nerves of the pelvic cavity]. In: *Atlas der Anatomie des Rindes: Lehrbuch für Tierärzte und Studierende. Schlütersche, 1st Edn* (Eds KD Budras & A Wünsche) pp. 84–87. Hannover, Germany

Burvenich C 1980 Variations of mammary artery blood flow and milk yield under normal conditions and during the oestrus cycle of the dairy goat. *Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde* **43** 18–27

Burvenich C, Paape MJ, Hoeben D, Dosogne H, Massart-Leen AM & Blum J 1999 Modulation of the inflammatory reaction and neutrophil

defence of the bovine lactating mammary gland by growth hormone. *Domestic Animal Endocrinology* **17** 149–159

Christensen K, Nielsen MO, Bauer R & Hilden K 1989 Evaluation of mammary blood flow measurements in lactating goats using the ultrasound Doppler principle. *Comparative Biochemistry and Physiology* **92A** 385–392

Davis SR & Collier RJ 1985 Mammary blood flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68** 1041–1058

De Mol RM & Ouweltjes W 2001 Detection model for mastitis in cows milked in an automatic milking system. *Preventive Veterinary Medicine* **49** 71–82

Dhondt G, Burvenich C & Peeters G 1977 Mammary blood flow during experimental *Escherichia coli* endotoxin induced mastitis in goats and cows. *Journal of Dairy Research* **44** 433–440

Dosogne H, Burvenich C, van Weren T, Roets E, Noordhuizen-Stassen EN & Goddeeris B 1997 Increased surface expression of CD11b receptors on polymorphonuclear leucocytes is not sufficient to sustain phagocytosis during *Escherichia coli* mastitis in early postpartum dairy cows. *Veterinary Immunology and Immunopathology* **60** 47–59

Grunert E (1990): [Udder]. In: *Die klinische Untersuchung des Rindes 3rd Edn.* (Ed. G Rosenberger) p. 525. Berlin and Hamburg, Germany: Verlag Paul Parey

Hollis B, Mavrides E, Campbell S, Tekay A & Thilaganathan B 2001 Reproducibility and repeatability of transabdominal uterine artery Doppler velocimetry between 10 and 14 weeks of gestation. *Ultrasound in Obstetrics and Gynecology* **18** 593–597

Houvenaghel A, Peters G & Verschooten F 1973 Influences of manual udder stimulation and oxytocin on mammary blood flow in lactating cows. *Archives Internationales de Pharmacodynamie et de Thérapie* **205** 124

Krueger L, Koerte J, Tsousis G, Herzog K, Flachowsky G & Bollwein H 2009 Transrectal Doppler sonography of uterine blood flow during the first 12 weeks after parturition in healthy dairy cows. *Animal Reproduction Science* **114** 23–31

Lamb CR, Burton CA & Carlisle CH 1999 Doppler measurements of hepatic arterial flow in dogs: technique and preliminary findings. *Veterinary Radiology and Ultrasound* **40** 77–81

Lough DS, Beede DL & Wilcox CJ 1990 Effects of feed intake and thermal stress on mammary blood flow and other physiological measurements in lactating dairy cows. *Journal of Dairy Science* **73** 325–332

Meurer DG 1999 [Stages of the inflammation]. In: *Allgemeine Pathologie: Kompendium für die Veterinärmedizin* pp. 118–119. Schattauer Stuttgart, Germany: Verlagsgesellschaft mbH

Miller GY, Bartlett PC, Lance SE, Anderson J & Heider LE 1993 Costs of clinical mastitis and mastitis prevention in dairy herds. *Journal of the American Veterinary Medical Association* **A 202** 1230–1236

Petzl W, Zerbe H, Günther J, Yang W, Seyfert H-M, Nürnberg G & Schuberth H-J 2008 *Escherichia coli,* but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Veterinary Research* **39** 18

Piccione G, Arcigli A, Fazio F, Giudice E & Caola G 2004a Pulsed wave-doppler ultrasonographic evaluation of mammary blood flow speed in cows during different productive periods. *Acta Scientiae Veterinariae* **32** 171–175

Piccione G, Arcigli A, Assenza A, Percipalle M & Caola G 2004b Pulsed wave-Doppler ultrasonographic evaluation of the mammary blood flow in the ewe. *Acta Veterinaria* **73** 23–27

Prakash BS, Meyer HHD, Schallenberger E & van de Wiel DFM 1987 Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the antibody technique. *Journal of Steroid Biochemistry* **28** 623–627

Rajala-Schultz PJ & Gröhn YT 1999 Culling of dairy cows. Part I. Effects of diseases on culling in Finnish Ayrshire cows. *Preventive Veterinary Medicine* **41** 195–208

Rauch A, Krueger L, Miyamoto A & Bollwein H 2008 Color Doppler sonography of cystic ovarian follicles in cows. *Journal of Reproduction and Development* **54** 447–453

Schmucker N, Schatzmann U, Budde K, Gundel M, Jäggin CE & Meier H 2000 Duplex-ultrasonographic evaluation of the common carotid artery in the resting, sedated and anesthetized horse. *Veterinary Radiology and Ultrasound* **41** 168–171

Seegers H, Fourichon C & Beaudeau F 2003 Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* **34** 475–491

Vanselow J, Yang W, Herrmann J, Zerbe H, Schuberth HJ, Petzl W, Tomek W & Seyfert HM 2006 DNA-remethylation around a STAT5-binding enhancer in the alphaS1-casein promoter is associated with abrupt shutdown of alpha-s_1-casein synthesis during acute mastitis. *Journal of Molecular Endocrinology* **37** 463–477

6. Discussion

6.1 Establishment of a new non-invasive method for monitoring mammary blood flow in cows

Basically, this part of the study was divided into an initial adaption phase and a subsequent experimental phase. The aim was to establish transrectal colour Doppler sonography (TCDS) as a non-invasive and precise method for evaluating the physiological blood flow of the pudendoepigastric trunk in udder-healthy cows.

Adaption phase: In a three week adaption phase test animals were habituated to their new environment and to the unaccustomed scanning procedure for avoiding stressful situations during the experimental phase. The avoidance of stress is a basic demand of correct Doppler sonographic measurements.

It is known, that adrenaline and noradrenaline released during stressful situations cause a sudden and marked reduction in mammary blood flow, because of vasoconstriction. For goats it is described, that moments of enhanced stress can lower udder blood flow to half and recovery to initial values takes a half to one hour (Linzell & Rasmussen 1972). Therefore, exact evaluations of mammary BFV would not be possible being carried out during stressful situations.

Experimental phase: To minimise or eliminate disturbing variables possibly influencing the results, the main focus of the trial design was on an initially large homogeneity of the animals (number of lactations, stage of lactation, health status, SCC, bacteriological status of the milk). The compliance with very stringent criteria applying to the animals was seen as an absolute precondition for justifying the use of only five animals.

Choice of the adequate scanning location: Doppler sonographic measurements of the bovine mammary blood flow were carried out scanning the major supplier of the mammary gland of cows, the transrectally assessable left and right pudendoepigastric trunk. This location complies with the requirements of providing reliable measurement data as scannings of this vessel section keep the general principles of Doppler sonographic measurements (angle of insonation; non-affected vessel lumen) and cause only a little irritation of the examined animals.

A brief trial scanning the blood flow of the much more easily accessible dorsal labial branch and the subcutaneous abdominal vein (data not shown) immediately showed the infeasibility of these two scanning locations.

The thin dorsal labial branch lying on the caudal body surface does not have the potential for providing reliable results due to very winding runs of the vessel and an extreme irritation of the animals by contact. Every movement of the face of the ultrasound transducer on the skin causes titillation followed by disturbing defence reactions of the examined animals. Sedations with xylazine hydrochloride for calming down the animals are out of question because of decreasing (Braun & Föhn 2005) and, thus, adulterant effects on mammary blood flow. A practical use of this scanning location is not present.

The subcutaneous abdominal vein is also not considered to be an adequate scanning location for measuring mammary blood flow changes since conclusions about the actual mammary (arterial) influx cannot be drawn. Moreover, exact Doppler sonographic measurements of this very raised and relocatable blood vessel are nearly impossible with no sufficiently buffering tissue between the vascular wall and the face of the ultrasound transducer. The risk of erroneous measurements by narrowing the vessel lumen is remarkable. Furthermore, the problem of animal irritation by contact is present, too.

Choice of the adequate blood flow parameter: According to former studies measuring mammary blood flow in cows (Kensinger et al. 1983; Gorewit et al. 1989; Thivierge et al. 2000; Delamaire & Guinard-Flament 2006; Honnens et al. 2007) and goats (Christensen et al.

1989) by different methods BFV was used as the parameter of choice. Thereby, aspects of clearness and comparability were in the spotlight. Also, in other bovine (Krueger et al. 2009) or equine (Bollwein et al. 2003) genital organs BFV was prevalently evaluated.

In the beginning of this trial the intra-observer precision of this new method was necessarily assessed and possible dependences on disturbance variables (left/right; pre-/post-milking; oestrus/dioestrus) were tested. In literature several methods of evaluating intra-observer precision are described (Lamb et al. 1999; Bollwein et al. 2000; Schmucker et al. 2000). In the present study measurements were taken three times on each side and precision was expressed by the CV and the ICC similar to other studies (Bollwein et al. 2000; Hollis et al. 2001). The intra-observer precision of repeatedly performed measurements in single animals was consistently good. The overall mean, using left and right measurements of all five animals, also supported this finding with a CV of 8.3 %. It was demonstrated that TCDS of the pudendoepigastric trunk provides well reproducible and, thus, precise values.

On the basis of former studies using Doppler sonography for different investigations, bilateral comparisons were drawn for information about relevancies and differences of the two scanning locations, the left and the right pudendoepigastric trunk (Christensen et al. 1989; Bollwein et al. 1998; Schmucker et al. 2000; Bollwein et al. 2002; Piccione et al. 2004a). A significant difference between measurements of the left and the right pudendoepigastric trunk was not found, however, the animal number in the present study was limited. Yet, a power analysis using the standard deviation of 0.5, which was found in the present study, calculated that a difference in the BFV of at least 1 l/min between left and right would have been identified with a power of 90% and alpha = 0.05 using the five animals (calculated with NCSS/PASS). This indicates similar blood support of both udder halves and equates with almost equally distributed MY of the left and the right udder halves (data not shown) when kept under physiological conditions like in this trial. The results also corroborate findings of other authors using Doppler ultrasound for measurements of mammary blood flow velocities at different udder-associated vessels in lactating cows (Piccione et al. 2004a), goats (Christensen et al. 1989) and ewes (Piccione et al. 2004b).

A significant difference between measurements before and after milking could not be discovered. Similar BFV observations are reported for mammary blood flow measurements in lactating goats (Christensen et al. 1989). Former studies using invasive methods at the external pudic artery in cows showed that positive changes of BFV are only measurable during milking. An abrupt and enormous increase of mammary blood flow with onset of milking followed by a very quick return of BFV within minutes to initial values at the end of milking has been described (Houvenaghel et al. 1973; Davis & Collier 1985). This can be seen as an explanation for not detecting any differences between pre- and post-milking measurements within two hours in the present trial.

From goats (Burvenich 1980) and cattle (Bollwein et al. 2000) it is known that oestrus cycle affects the blood flow in certain blood vessels. In goats the mammary BFV is decreased the day before and the day of oestrus, but no alteration is seen on any other days during the oestrus cycle (Burvenich, 1980). In the present trial a significant difference of BFV between scannings during oestrus and dioestrus was not detected.

With this recent study it was successfully shown that TCDS is a new non-invasive and precise method for evaluating the BFV of the pudendoepigastric trunk in healthy cows.

The stated discoveries mark an important basis for further investigations of the mammary blood flow, especially for experimental setups of infection models with udder pathogens and the associated detection of clinical, acute / chronic or subclinical mastitis by TCDS. Findings of this study provide significant information for bilateral comparisons and scanning times in mastitis infection models and should be seen as a helpful guideline for planned Doppler sonographic testings.

It has to be noted that only initially udder-healthy animals with an equally distributed MY in both udder halves were examined in the present trial. Animals with preexisting impairments of the udder or such with unequal milk distribution in the udder halves could have biased the results of the standardised study. This fact underlines the particular meaning and suitability of the findings described here with regards to well-defined mastitis infection models. Setting a

"zero-standard", meaning healthy-, left / right- and hour 0-status, of the individual test animal before trial initiation is an absolute prerequisite for any examination.

6.2 TCDS – a method for early mastitis detection?

Bovine mastitis is one of the most significant diseases in dairy herds with huge effects on farm economics due to milk losses and treatment costs during current and / or subsequent lactations. Especially intramammary infections with *E. coli* very often lead to an acute mastitis with grave clinical effects (Bannermann et al. 2004). The importance of an early detection of inflammatory events related to the mammary gland of cows is obvious.

Unfortunately, traditional mastitis detection methods such as clinical examination (palpation of the udder, RT, assessment of the milk secretion), CMT (estimation of SCC) or BE (culturing method) come into operation mainly at a very late stage of inflammation. Severe damages of the mammary tissue and, thus, a decelerated healing are the consequences. Novel assays under development such as measuring volatile components and potentiometric values of milk or new biosensor systems analysing lactose and EC show promise. However, EC is dependent on several factors (stage of lactation, food and breed) and, thus, still not sufficiently precise.

All of these methods have in common that they are milk-related. Detection of an inflammation of the bovine gland during the dry period or in heifers is not possible.

It is known that *E. coli* is able to persist in the udder during the dry period (Bradley & Green 2001) and it is assumed that more than half of all *E. coli* mastitides occurring within the first two months of lactation are related to infections during the dry period (Smith et al. 1985). In consideration of these facts, new technologies for early mastitis detection independent from the act of milking have to be developed.

Though the relatively new technique of infrared thermography is non-milk-related, it is still under development and defective due to the strong influence of ambient temperatures (Viguier et al. 2009).

Former studies have shown that TCDS is an established non-invasive and precise method for evaluating both physiological (Bollwein et al. 2002; Krueger et al. 2009) and pathological (Schmauder 2003; Rauch et al. 2008) changes of genital organs in cattle. For years, a close relationship between inflammatory processes of the mammary gland and mammary blood flow has been known (Dhondt 1977a). After its successful establishment in udder-healthy cows, as shown above, TCDS was now assumed also to be a non-milk-related, adequate technique for detecting possible pathological mammary blood flow changes at an early stage of *E. coli* mastitis.

At this part of the study a basic division into an initial adaption phase as described above and a subsequent experimental phase was made again. The same animals as in the "Establishment part" were used.

E. coli mastitis model: With regard to the aim of an investigation of mammary BFV changes at an early stage of an induced *E. coli* mastitis the need for an adequate mastitis model was obvious.

Over the past decades several mastitis models have been used for dealing with different problems. In animals fallen ill spontaneously starting conditions are hardly controllable and interpretable, whereas infection models allow deliberate specifications of those starting conditions. Thereby, nature and strictness of the model limits are crucial. However, in the past model requirements differed highly. In few cases test animals have been chosen according to strict criteria such as number of lactations, stage of lactation, low SCC and repeatedly bacteriologically negative milk samples (Schukken et al. 1999).

In this present mastitis model only udder-healthy animals with good general conditions were used. Moreover, the tested animals were of the same age, the same stage of lactation and synchronised in their stage of the oestrus cycle.

SCC is classified as an important parameter for the health status of the udder. A considerable increase of SCC during an *E. coli* mastitis is usual. Former studies have shown, that initially enhanced values of SCC lead to an attenuation of inflammation symptoms during natural as well as during experimentally induced mastitides (Nickerson et al. 1990; Shuster et al. 1996). For setting very strict model limits a maximum value of 50.000 cells / ml / udder quarter was required in this present study. To the author´s knowledge, up to now a mastitis model designed that way is unique.

Because of increasing cell counts related to increasing numbers of lactations as well as to durations of the lactation (Doggweiler & Hess 1983; Burvenich et al. 1994), only cows in their first mid lactation were used in this study. Preliminary mastitides of the animals were considered as knock-out criteria. Therefore, a compliance with these requirements is easier and more authentic for cows in their first lactation. Possibly latent infections could have lead to unscheduled mastitides initiated by exogenic stress factors (Wegner et al. 1976) and thus, to an incomparableness of the individual animals.

All animals used in this *E. coli* mastitis trial were synchronised in their stage of the oestrus cycle. For better reproducibility of the evaluated Doppler sonographic measurements during udder inflammation all test animals were inoculated with pathogens during oestrus. This particular time allows a precise specification of a period of 18 h. The advantage over other stages of the oestrus cycle is obvious.

Mastitis developments are known to be very differing due to influences of pathogen, host or environment factors (Park et al. 2004). Therefore, the assessment and documentation of clinical signs and their progresses are initially important. Only the occurance of visible alterations of the udder, the milk or the general condition indicates animals that have fallen ill. In this present model, clinical parameters were used for investigating temporal courses during an induced *E. coli* mastitis. Moreover, pathogen specific severities and systemic effects of the inflammation were characterised (Table 1).

In literature, different information about both infection periods and trial periods of induced *E. coli* mastitides are found. Ranges from 96 h (Kornalijnslijper et al. 2003) to 21 days (Lohuis et al. 1990) are described. In this present mastitis model, acute udder inflammations and their effects on mammary blood flow at an early stage of *E. coli* mastitis were focused upon. The time period up to 24 h p.i. was defined as the early stage of a definitely established *E. coli* mastitis.

In this trial mastitides caused by *E. coli* were characterised by acute courses with moderate to severe clinical signs (Table 1) according to former studies (Burvenich et al. 2003). Invariantly successful infections were corroborated in all test animals on the basis of clinical findings. Remarkable swellings and increased firmnesses caused by subcutaneous and interstitial oedemas were observed for all inoculated udder quarters at the time of 24 h p.i. Milk secretion of the infected quarters of all animals showed huge amounts of milk flakes and milk character was absent in four animals.

After pathogen inoculation a significant difference of BFV in the right infected udder half between 0 und 12 h p.i. was detected. The first change of BFV can be easily explained by the basic principles of inflammation. After contact with the pathogen processes regulated by biochemical mediators provoke a dilatation of the arterioles, capillaries and venules. Increased hydrostatic pressure affects transudation and hyperaemia (Meurer, 1999). An increased volume of blood is moved into the mammary region.

In a former study, measuring mammary blood flow by an implanted electromagnetic flow probe after intramammary infusion of *E. coli* endotoxin two blood flow peaks were found (Dhondt et al. 1977a). A first peak occurred at the third hour after infusion followed by a temporary return to the initial level and a second peak between the tenth and eleventh hour followed by a return to control levels between the thirteenth and fourteenth hour. Since, in our study, measurements were made at times 0, 12 and 24 h p.i. detection of the described first blood flow peak was not possible. However, the described second peak could be entirely validated by TCDS.

Similar observations are known from induced metritides in cows (Schmauder 2003). Already one hour after an intrauterine infusion of 100ml of 4% Lotagen® (metacresolsulphonic acid and formaldehyde condensation product) an increase of nearly 70% of the uterine blood flow velocity was evaluated followed by a temporary return to initial values six hours after infusion. The blood flow velocity increased again at the twelfth hour and stayed on an enhanced level until the third day after Lotagen® infusion.

In the present trial significant differences of simultaneously increasing RT and SCC in the infected quarter between 0 and 12 h p.i. also fit well into the inflammatory process with systemic and cellular defence mechanisms during the acute phase. Thereby, the statistically positive correlation between the changes of the right SCC and the changes of the right BFV clarifies well the degree of acute inflammation and its effect on mammary blood flow.

The notable increase of RT of all animals between 0 and 12 h p.i. goes along with findings of other studies dealing with *E. coli* mastitis (Bannermann et al. 2004). Interestingly, only a mild to moderate impairment of the general condition was observed (Table 1). This phenomenon can be explained by the use of test animals being in mid lactation. A decreasing severitiy of clinical alterations is known during proceeded stages of lactation, unlike during the postpartal phase (Burvenich et al. 2003).

A significant difference of BFV between the right (infected) and the left (non-infected) side at the time of 12 h p.i. is remarkable because it allows the exact identification of the infected udder half.

According to former studies (Lohuis et al. 1990) a considerable decline of MY, also in the uninfected udder quarters (Shuster et al. 1996), was detected. Although the decrease of the MY of the right infected udder half was not statistically significant, the change of the MY correlated positively with the change of the increasing right BFV.
At this stage of experimental *E. coli* mastitis it is known that mammary tissue shuts down synthesis of milk constituents and favours the expression of antimicrobial effector molecules like defensins (Vanselow et al. 2006, Petzl et al. 2008). This could explain the increase of the BFV up to 12 h p.i. due to proinflammatory actions, although the MY is already decreasing.

Also a temporary interruption of food intake can be seen as an explanation for a decreasing total (left and right udder quarters) MY. It is known, that nutritional deficiencies do have negative short-term effects on the MY (Lohuis et al. 1988).

From lactating goats it is known that intramammary infusions of colchicine lead to a distinct decrease of MY, but to a maintenance or even an increase of mammary blood flow (Henderson & Peaker 1980) associated with pyrexia and a local inflammatory response (Burvenich & Peeters 1980).

Between 12 and 24 h p.i. the BFV of the right pudendoepigastric trunk (infected side) decreased significantly accompanied by significantly decreasing RT and MY (right) and a significant increasing SCC (right). These findings also follow the basic inflammatory principles: Several hours after the occurrence of inflammation the dilatation of arterioles and the arterial branch still continues, whereas the venous branch and small veins start to constrict. Hyperaemia and a slowdown of blood flow are the consequences (Meurer, 1999). A lower volume of blood is moved into the mammary region. After the acute phase of inflammation RT returns to a lower level.

On one hand the significant decrease of MY of the right infected side can be explained by the ongoing progression of the systemic illness which influences, typically for *E. coli* mastitis, also the MY of the left non-infected side (Burvenich et al. 1999). A high correlation between decreasing MY of the uninfected gland and bacterial growth of the infected gland is known (Dosogne et al. 1997). On the other hand, interestingly, not only the infected right udder half showed an increased SCC as expected, but also the non-infected left udder half presented a significantly increased SCC between the times 0 and 24 h p.i. The migration of leukocytes not only into tissue of the infected side of the gland, but also to the contralateral side might be an explanation for this phenomenon (Kimura et al. 2005).
It is also described that an acute mastitis influences the cellular response of neighbouring udder quarters (Petzl et al. 2008).

In conclusion the *E. coli* mastitis model used in this trial can be described as an experimental setup providing well reproducible results. The clinical courses of inflammation absolutely equated to an acute *E. coli* mastitis under field conditions in mid lactation.

6.3 Future prospects

With this present study it could not only be shown that TCDS is a new non-invasive and precise method for evaluating the BFV of the pudendoepigastric trunk in healthy cows, but that this technique also was successfully used for detecting pathological mammary blood flow changes at an early stage in a stringent *E. coli* mastitis model.

It is clear that TCDS at the present time does not provide a practical and realistic option for a routine early detection of *E. coli* mastitis. But especially with a view to the severe problem of mastitis appearing worldwide and the need to detect it as early as possible, it is conceivable that TCDS may find its way from an experimental technique to a clinical application as another useful tool for diagnosing pathological changes in the mammary gland of cows. However, more studies including some on the economical and logistical practicability of the method are needed.

But moreover, it is obvious that TCDS is a real alternative to invasive methods measuring mammary blood flow in cows. In consideration of the fact that only little is known about relations between mammary blood flow and mammary metabolism and their reciprocal regulation mechanisms, further research on this field is needed. For that purpose TCDS is seen as an adequate technique for investigating nutritional and metabolic effects on bovine mammary BFV in an easy and precise manner with a low impact on the examined animals.

7. Summary

Bovine mastitis is still ranked among the main production diseases in dairy herds of developed countries. Particularly intramammary infections with *Escherichia coli (E. coli)* very often lead to acute mastitides with severe clinical effects. Enormous economic losses during current and / or subsequent lactations are the consequences. Although the identification of inflammatory events at an early stage is a basic demand of milk hygiene and an essential precondition for a successful therapy, no sufficient methods for an early mastitis detection are available to date.

In this present investigation transrectal colour Doppler sonography (TCDS) was initially established as a non-invasive and precise method for examining the physiological blood flow of the pudendoepigastric trunk, a rectally accessible vessel section of the udder´s main supplier in cows, and subsequently used for detecting pathological mammary blood flow changes in a standardized *E. coli* mastitis model with so far unavowedly strict animal criteria.

Five primiparous Holstein dairy cows, four to six months after calving, were examined in two trials: a methodological part and an experimental part of the study. All monitored udder quarters were initially clinically healthy, somatic cell count (SCC) <50 000 cells/ml and milk samples bacteriologically negative. The blood flow of the left and the right pudendoepigastric trunk was described by the blood flow volume (BFV).

In the methodological part of the study, the intra-observer precision of the method was evaluated. The coefficients of variation of the BFV were 7.1% (6.8 +/- 2.6 l/min) for the left and 9.4% (6.8 +/- 1.3 l/min) for the right pudendoepigastric trunk, an average of 8.3% (6.8 +/- 1.9 l/min). The intraclass correlation coefficients of the BFV were 0.99 ($p<0.001$) for the left and 0.75 ($p=0.004$) for the right vessel. BFV did not differ significantly between the left and the right side ($p=0.893$) nor between pre- and post-milking (left side: $p=0.345$; right side: $p=0.345$) nor between oestrus and dioestrus (left side: $p=0.690$; right side: $p=0.893$).

In the experimental part of the study, significant differences of increasing BFV between 0 and 12 h p.i. (post infectionem) ($p=0.043$) and decreasing BFV between 12 and 24 h p.i. ($p=0.043$) were discovered for the pudendoepigastric trunk of the infected right side. No significant differences were detected for the left non-infected side ($p>0.05$). In the left / right – comparison (control / infection) a significant increase of the right BFV was observed at 12 h p.i. ($p=0.043$). A significant increase of the rectal temperature could be detected between 0 and 12 h p.i. ($p=0.043$) as well as a statistically significant decrease between 12 and 24 h p.i. ($p=0.043$). Between 12 and 24 h p.i. a significant decrease of the milk yield was seen in the infected right udder half ($p=0.042$). The increasing SCC of the right udder quarters showed significant differences between 0 and 12 h p.i. ($p=0.043$) as well as between 12 and 24 h p.i. ($p=0.043$). A significant difference of the SCC was observed in the left-right comparison at the time of 24 h p.i. ($p=0.043$). The difference of an increasing SCC correlated positively with the difference of an increasing BFV between 0 and 12 h p.i. (Spearman's $\rho=1.00$; $p=0.043$) for the right infected side. Between 12 and 24 h p.i. the difference of an increasing SCC correlated negatively with the difference of a decreasing BFV ($\rho=-0.60$; $p=0.043$) for the right pudendoepigastric trunk.

The present study showed TCDS as a new non-invasive and precise method for evaluating the physiological BFV of the pudendoepigastric trunk in cows. It also demonstrated the successful use of this technique for detecting pathological mammary blood flow changes at an early stage of an acute *E. coli* mastitis.

8. Zusammenfassung

Die Mastitis des Rindes zählt immer noch zu den wichtigsten Erkrankungen in modernen Milchviehbeständen. Vor allem intramammäre Infektionen mit *Escherichia coli (E. coli)* verursachen sehr oft akute Mastitiden mit schweren klinischen Verläufen. Erhebliche wirtschaftliche Verluste während aktueller und / oder folgender Laktationen sind die Konsequenz. Obwohl die rasche Erkennung entzündlicher Ereignisse eine Grundvoraussetzung der Milchhygiene und der erfolgreichen Therapie darstellt, sind aktuelle Methoden zur Mastitis-Früherkennung immer noch unbefriedigend.

In der hier vorliegenden Untersuchung wurde die transrektale Farbdopplersonographie zunächst als nicht-invasive und präzise Methode zur Messung physiologischer Blutflüsse des Truncus pudendoepigastricus, eines rektal erreichbaren Gefäßabschnittes des Euterhauptversorgers, in Kühen etabliert und anschließend zur Beobachtung pathologischer mammärer Blutflußänderungen in einem standardisierten *E. coli* Mastitismodell mit außerordentlich strengen Tieranforderungen und Modellgrenzen eingesetzt.

Fünf primipare Kühe der Rasse Holstein-Friesian, vier bis sechs Monate post partum, wurden in zwei getrennten Versuchsteilen, einem methodischen Teil und einem experimentellen Teil, untersucht. Alle beobachteten Euterviertel waren zu Beginn klinisch gesund, die Zellzahlen (SCC) pro Euterviertel unter 50 000 Zellen/ml und gewonnene Milchproben bakteriologisch negativ. Der Blutfluß des rechten und linken Truncus pudendoepigastricus wurde durch das Blutflußvolumen (BFV) beschrieben.

Im methodischen Teil der Studie wurde zunächst die Präzision „innerhalb“ eines Untersuchers (intra-observer precision) bestimmt. Die Variationskoeffizienten des BFV waren 9,4% (6,8 +/- 1,3 l/min) für den rechten und 7,1% (6,8 +/- 2,6 l/min) für den linken Truncus pudendoepigastricus, im Durchschnitt 8,3% (6,8 +/- 1,9 l/min). Die Intraklassen-Korrelationskoeffizienten des BFV waren 0,75 ($p=0,004$) für das rechte und 0,99 ($p<0,001$) für das linke Blutgefäß. Weder zwischen der rechten und der linken Seite ($p=0,893$), noch zwischen vor und nach dem Melken (rechte Seite: $p=0,345$; linke Seite: $p=0,345$), noch

zwischen Östrus und Diöstrus (rechte Seite: p=0,893; linke Seite: p=0,690) unterschied sich das BFV statistisch signifikant.

Im experimentellen Teil der Studie wurden am Truncus pudendoepigastricus der infizierten rechten Seite signifikante Unterschiede des zunehmenden BFV zwischen 0 und 12 h p.i. (post infectionem) (p=0,043) und des abnehmenden BFV zwischen 12 und 24 h p.i. (p=0,043) festgestellt. Die nicht infizierte linke Seite zeigte keine signifikanten Unterschiede (p>0,05). Im rechts / links - Vergleich (Infektion / Kontrolle) wurde ein signifikanter Anstieg des rechten BFV zum Zeitpunkt 12 h p.i. (p=0,043) beobachtet. Sowohl ein signifikanter Anstieg der rektalen Temperatur zwischen 0 und 12 h p.i. (p=0,043) als auch ein statistisch signifikanter Abfall der rektalen Temperatur zwischen 12 und 24 h p.i. (p=0,043) wurden verzeichnet. Zwischen 12 und 24 h p.i. sank die Milchleistung der infizierten rechten Euterhälfte statistisch signifikant (p=0,042). Der Zellzahlanstieg der rechten Euterviertel zeigte signifikante Unterschiede sowohl zwischen 0 und 12 h p.i. (p=0,043) als auch zwischen 12 und 24 h p.i. (p=0,043). Ein signifikanter Unterschied der Zellzahl wurde im rechts / links - Vergleich zum Zeitpunkt 24 h p.i. (p=0,043) festgestellt. Zwischen 0 und 12 h p.i. korrelierte die Differenz der auf der infizierten rechten Seite ansteigenden Zellzahl positiv mit der Differenz des auf der infizierten rechten Seite ansteigenden BFV (Spearman's ρ=1,00; p=0,043). Zwischen 12 und 24 h p.i. korrelierte die Differenz der ansteigenden Zellzahl der infizierten rechten Seite negativ mit der Differenz des sinkenden BFV der rechten Seite (ρ=-0,60; p=0,043).

In der vorliegenden Studie wurde die transrektale Farbdopplersonogphie als eine neue, nicht-invasive und präzise Methode zur Bestimmung physiologischer Blutflußvolumina des Truncus pudendoepigastricus in Kühen etabliert. Darüber hinaus konnte die erfolgreiche Anwendung dieser Technik zur Erkennung pathologischer mammärer Blutflußänderungen zu einem frühen Zeitpunkt einer akuten *E. coli* Mastitis gezeigt werden.

9. References

Acland HM 1995 Mammary Gland. In: Carlton WW, McGavin MD (eds.) THOMSON´S SPECIAL VETERINARY PATHOLOGY. Mosby-Year Book, Inc., 2nd edn, St. Louis, USA, 537

ADR 2007 [Reasons for leaving herd]. In: Rinderproduktion in der Bundesrepublik Deutschland 2007. Verlag ADR, Bonn, Germany, Table 4.13

Bannermann DD, Paape MJ, Lee JW, Zhao X, Hope JC, Rainard P 2004 *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clinical and diagnostic laboratory immunology **11** 463-472

Barrow PA & Hill AW 1989 The virulence characteristics of strains of *Escherichia coli* isolated from cases of bovine mastitis in England and Wales. Veterinary microbiology **20** (1) 35-48

Bauman DE, McCutcheon SN 1985 The effects of growth hormone and prolactin on metabolism. In: Milligan LP, Grovum WL, Dobson A (Ed.) Proc. VI Int. Symp. On Ruminant Physiology: control of Digestion and Metabolism in Ruminants. Ch 23. Reston Publ.Co., Reston VA

Baumgartner UM 1998 [Coulor Doppler sonography of the A. uterina and the Corpus luteum in cows]. Ludwig-Maximilians-University Munich, Germany diss

Bollwein H, Maierl J, Mayer R, Stolla R 1998 Transrectal color Doppler sonography of the A. uterina in cyclic mares. Theriogenology **49** 1483-1488

Bollwein H, Meyer HHD, Maierl J, Weber F, Baumgartner U, Stolla R 2000 Transrectal Doppler sonography of uterine blood flow in cows during the estrous cycle. Theriogenology **53** 1541-1552

Bollwein H, Baumgartner U, Stolla R 2002 Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. Theriogenology **57** 2053-2061

Bollwein H, Weber F, Woschée I, Stolla R 2004 Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. Theriogenology **6** 499-509

Bradley AJ, Green MJ 2001 Adaptation of *Escherichia coli* to the bovine mammary gland. Journal of clinical microbiology **39** 1845-1849

Bragulla H, König HE 1999 [Mammary gland]. In: König HE, Liebich HG (eds.), Anatomie der Haussäugetiere: Lehrbuch und Farbatlas für Studium und Praxis Bd. II. F. K. Schattauer Verlagsgesellschaft mbH, 1st Edn Stuttgart, Germany, 343

Braun U, Föhn J 2005 Duplex ultrasonography of the common carotid artery and external jugular vein of cows. American journal of veterinary research **66** 962-965

Braun U, Hoegger R 2008 B-mode and colour Doppler ultrasonography of the milk vein in 29 healthy Swiss braunvieh cows. The veterinary record **163** 47-9

Breen JE, Green MJ, Bradley AJ 2009 Quarter and cow risk factors associated with the occurrence of clinical mastitis in dairy cows in the United Kingdom. Journal of dairy science **92** 2551-2561

Brolund, L 1985 Cell counts in bovine milk. Causes of variation and applicability for diagnosis of subclinical mastitis. Acta veterinaria Scandinavica **80** 114–123

Budras KD, Wünsche A 2002 [Arteries, veins and nerves of the pelvic cavity]. In: Budras KD, Wünsche A (eds.), Atlas der Anatomie des Rindes: Lehrbuch für Tierärzte und Studierende. Schlütersche, 1st Edn Hannover, Germany, 84-87

Bühlmeyer M 1999 [Colour Doppler sonography of the A. ovarica in ewes during spontaneous and hormonally induced ovulations]. Ludwig-Maximilians-University Munich, Germany diss

Burvenich C 1980 Variations of mammary artery blood flow and milk yield under normal conditions and during the oestrus cycle of the dairy goat. Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde **43** 18-27

Burvenich C, Peeters G 1980 Effect of intramammary infusion of colchicine on mammary blood flow in lactating goats. Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde **44** 211

Burvenich C, Paape MJ, Hill AW, Guidry AJ, Miller RH, Heynemann R, Kremer WD, Brand A 1994 Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. The Veterinary quarterly **16** 45-50

Burvenich C, Paape MJ, Hoeben D, Dosogne H, Massart-Leen AM, Blum J 1999 Modulation of the inflammatory reaction and neutrophil defense of the bovine lactating mammary gland by growth hormone. Domestic Animal Endocrinology **17** 149-159

Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A, Duchateau L 2003 Severity of *E. coli* mastitis is mainly determined by cow factors. Veterinary research **34** 521-564

Chaiyabutr N, Faulkner A, Peaker M 1980 Effect of starvation on the cardiovascular system, water balance and milk secretion in lactating goats. Research in veterinary science **28** 291

China B, Goffaux F 1999 Secretion of virulence factors by *Escherichia coli*. Veterinary research **30** 181-202

Christensen K, Nielsen MO, Bauer R, Hilden K 1989 Evaluation of mammary blood flow measurements in lactating goats using the ultrasound Doppler principle. Comparative biochemistry and physiology **92A** 385-392

Colak A, Polat B, Okumus Z, Kaya M, Yanmaz LE, Hayirli A 2008 Short communication: early detection of mastitis using infrared thermography in dairy cows. Journal of dairy science **91** 4244-4248

Culina M, Hahne J, Vorlop K-D 2006 Design of an online sensor array for an early detection of udder affections in automatic milking systems. In: World Congress of Agricultural Engineering for a Better World: Book of Abstracts. VDI Verlag GmbH, Bonn, Germany, 453-454

Cvek K 1997 Mammary gland function with special reference to vascularisation and atrial natriuretic peptide. Swedish University of Agricultural Sciences, Uppsala, Sweden diss

Davis SR, Collier RJ, McNamara JP, Head HH 1983 Effect of growth hormone and thyroxine treatment of dairy cows on milk production, cardiac output and mammary blood flow. Proceedings of the Endocrine Society of Australia **26** 31

Davis SR, Collier RJ 1985 Mammary Blood Flow and Regulation of Substrate Supply for Milk Synthesis. Journal of dairy science **68** 1041-1058

Davis SR, Collier RJ, McNamara JP, Head HH, Sussman W 1988 Effects of thyroxine and growth hormone treatment of dairy cows on milk yield, cardiac output and mammary blood flow. Journal of animal science **66** 70-79

Deane C 1995 Doppler ultrasound: physical principles. In: Arnold E (eds.): A Colour Atlas of Doppler Sonography in Obstetrics. Harrington, K. a. S. Campbell, London, England 1-15

Delamaire E, Guinard-Flament J 2006 Increasing milking intervals decreases the mammary blood flow and mammary uptake of nutrients in dairy cows. Journal of dairy science **89** 3439-3446

Deluyker HA 1991 Milk yield fluctuations associated with mastitis. In: Burvenich C, Vandeputte-Van Messom G, Hill AW (Eds.) New insights into the pathogenesis of mastitis, Flemish Veterinary Journal, Merelbeke, Belgium 207-216

De Mol RM, Ouweltjes W 2001 Detection model for mastitis in cows milked in an automatic milking system. Preventive veterinary medicine **49** 71-82

De Schepper S, De Ketelaere A, Bannermann DD, Paape MJ, Peelman L, Burvenich C 2008 The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of Escherichia coli mastitis in dairy cattle. Veterinary research **39** 1-23

Dhondt G, Houvenaghel A, Peeters G, Verschooten F 1973 Influence of vasoactive hormones on blood flow through the mammary artery in lactating cows. Archives internationales de pharmacodynamie et de thérapie **204** 89-104

Dhondt G, Burvenich C, Peeters G 1977a Mammary blood flow during experimental *Escherichia coli* endotoxin induced mastitis in goats and cows. Journal of Dairy Research **44** 433-440

Dhondt G, Houvenaghel A, Peeters G, Jöchle W 1977b Effect of prostaglandins $F_{2\alpha}$ and E_2 on milk ejection, blood pressure and blood flow through the mammary artery in the cow. Prostaglandins **13** 1185-1199

Dickey RP 1997 Doppler ultrasound investigation of uterine and ovarian blood flow in infertility and early pregnancy. Human reproduction update **3** 467-503

Doggweiler R, Hess E 1983 [Cell count in the milk of unoffended udders]. In: Milchwissenschaft **38** 5-8

Dosogne H, Burvenich C, van Weren T, Roets E, Noordhuizen-Stassen EN, Goddeeris B 1997 Increased surface expression of CD11b receptors on polymorphonuclear leucocytes is not sufficient to sustain phagocytosis during *Escherichia coli* mastitis in early postpartum dairy cows. Veterinary immunology and immunopathology **60** 47-59

Dudwiesus H, Krieg R, Schmidt KJ 1993 [Physical-technical basic principles of Doppler sonography]. In: Sohn C, Stolz W, Bastirt G (eds.): Dopplersonographie in der Gynäkologie und Geburtshilfe. Georg Thieme Verlag, Stuttgart, Germany 1-11

Elbers AR, Miltenburg JD, De Lange D, Crauwels AP, Barkema HW, Schukken YH 1998 Risk factors for clinical mastitis in a random sample of dairy herds from the southern part of the Netherlands. Journal of dairy science **81** 420–426

Erb RE, Goodwin MM, Morrison RA, Shaw AO 1952 Lactation Studies. II. Effect of Estrus. Journal of dairy science **35** 234-244

Errikson Å, Persson Waller K, Svennersten-Sjauna K, Haugen JE, Lundby F, Lind O 2005 Detection of mastitic milk using a gas-sensor array system (electronic nose). International dairy journal **15** 1193-1201

Fehlings PDK 2008 [Ill udders cost money]. In: Bayerisches Landwirtschaftliches Wochenblatt. **198** 22-24

Flückiger M 1997 [Basic principles of ultrasound diagnostics]. In: Braun U (eds.): Atlas und Lehrbuch der Ultraschalldiagnostik beim Rind. Verlag Paul Parey, Berlin, Germany 1-8

Gorewit RC, Aromando MC, Bristol DG 1989 Measuring bovine mammary gland blood flow using a transit time ultrasonic flow probe. Journal of dairy science **72** 1918

Gosling RG, King DH 1975 Ultrasonic angiology. In: Arteries and veins. Marcus AW, Adamson L (eds.). Churchill Livingstone, Edinburgh, Scotland 61-98

Goswamy & Steptoe 1988 Doppler ultrasound studies of the uterine artery in spontaneous ovarian cycles. Human reproduction **3** 721-726

Gravert HO 1983 [Function of the mammary gland]. In: Die Milch: Erzeugung, Gewinnung, Qualität. Eugen Ulmer GmbH & Co., Stuttgart, Germany, 143

Green MJ, Green LE, Cripps PJ 1996 Low bulk milk somatic cell counts and endotoxin-associated (toxic) mastitis. The Veterinary record **138** 305-306

Grunert E 1990 [Udder]. In: Rosenberger G, Die klinische Untersuchung des Rindes. Verlag Paul Parey, 3rd Edn Berlin and Hamburg, Germany, 525

Hahn H, Falke D, Kaufmann S, Ullmann U 2005 [Medical microbiology and infectiology]. Springer Medizin Verlag, Ed Heidelberg SVB, Heidelberg, Germany

Hamann J, Fehlings K 2002 [Relevant aspects for combating bovine mastitis as a herd health problem]. In: Leitlinien zur Bekämpfung der Mastitis des Rindes als Bestandsproblem. Verlag der DVG Service GmbH, Gießen, Germany, 4, 11

Henderson AJ, Peaker M 1980 The effects of colchicine on milk secretion, mammary metabolism and blood flow in the goat. Quarterly journal of experimental physiology **65** 367-378

Heynemann R, Burvenich C, Vercauteren R 1990 Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced E coli mastitis. Journal of dairy science **73** 985-994

Hill AW 1991 Somatic cells – Friends or foes? In: Burvenich C, Vandeputte-Van Messom G, Hill AW (eds.). New insights into the pathogenesis of mastitis. Flemish Veterinary Journal, Merelbeke, Belgium 217-232

Hogan JS, Weiss WP, Todhunter DA, Smith KL, Schoenberger PS 1992 Efficacy of an Escherichia coli J5 mastitis vaccine in an experimental challenge trial. Journal of dairy science **75** 415-422

Hogan JS, Smith LK 2003 Coliform mastitis. Veterinary research **34** 507-519

Hollis B, Mavrides E, Campbell S, Tekay A, Thilaganathan B 2001 Reproducibility and repeatability of transabdominal uterine artery Doppler velocimetry between 10 and 14 weeks of gestation. Ultrasound in obstetrics & gynecology **18** 593-597

Honnens Ä, Goetze A, Herzog K, Bollwein H 2007 Assessment of mammarian blood flow during lactation in cows using transrectal doppler sonography. Reproduction in domestic animals **42** (Suppl. 1) 11

Houvenaghel A, Peters G, Verschooten F 1973 Influences of manual udder stimulation and oxytocin on mammary blood flow in lactating cows. Archives internationales de pharmacodynamie et de thérapie **205** 124

Hovinen M, Aisla AM, Pyörälä S 2006 Accuracy and reliability of mastitis detection with electrical conductivity and milk colour measurement in automatic milking. Acta agriculturæ Scandinavica. Section A, Animal science **56** 121-127

Hovinen M, Siivonen J, Taponen S, Hänninen L, Pastell M, Aisla AM, Pyörälä S 2008 Detection of clinical mastitis with the help of a thermal camera. Journal of dairy science **91** 4592-4598

Huszenicza G, Jánosi S, Gáspárdy A, Kulcsár M 2004 Endocrine aspects in pathogenesis of mastitis in postpartum dairy cows. Animal reproduction science **82-83** 389-400

Huth F-W 1995a [Milk synthesis]. In: Die Laktation des Rindes: Analyse, Einfluß, Korrektur. Eugen Ulmer GmbH & Co, Stuttgart, Germany, 10

Huth F-W 1995b [Milk synthesis]. In: Die Laktation des Rindes: Analyse, Einfluß, Korrektur. Eugen Ulmer GmbH & Co, Stuttgart, Germany, 11

Kensinger MH, Collier RJ, Wilcox CJ, Caton D 1983 Variability of resting mammary blood flow in nonlactating Holstein cows. Journal of dairy science **66** 1742-1746

Kimura K, Harp JA, Goff JP, Olsen SC 2005 Lymphocytes from one side of the bovine mammary gland migrate to the contra lateral gland and lymph node tissue. Veterinary immunology and immunopathology **108** 409-415

Kornalijnslijper JE, Beerda B, Daemen I, van der Werf J, van Werven T, Niewold T, Rutten V, Noordhuizen-Stassen E 2003 The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental *Escherichia coli* mastitis. Veterinary research **34** 721-736

Kremer WDJ, Noordhuizen-Stassen EN, Grommers FJ, Daemen AJJM, Brand A, Burvenich C 1993 Blood polymorphnuclear leukocyte chemotaxis during experimental *Escherichia coli* bovine mastitis. Journal of dairy science **76** 2613-2618

Krueger L, Koerte J, Tsousis G, Herzog K, Flachowsky G, Bollwein H 2009 Transrectal Doppler sonography of uterine blood flow during the first 12 weeks after parturition in healthy dairy cows. Animal reproduction science **114** 23-31

Lamb CR, Burton CA, Carlisle CH 1999 Doppler measurements of hepatic arterial flow in dogs: technique and preliminary findings. Veterinary radiology & ultrasound **40** 77-81

Leenanuruksa D, McDowell GH 1985 Effects of prolonged intravenous infusions of adrenaline on glucose utilization, plasma metabolites, hormones and milk production in lactating sheep. Australian journal of biological sciences **38** (2) 197-208

Linzell JL 1957 The measurement of udder blood flow in the conscious goat. Journal de physiologie **137** 75

Linzell JL 1960 Mammary gland blood flow and oxygen, glucose and volatile fatty acid uptake in the conscious goat. Journal de physiologie **153** 492

Linzell JL, Rasmussen F 1972 Mammary blood flow and changes in circulation during lactation in goats and cows. [2nd chapter]. In: Handbuch der Tierernährung II. Verlag Paul Parey, Hamburg und Berlin, Germany, 203-207

Linzell JL 1974 Mammary blood flow and methods of identifying and measuring precursors of milk. In: Lactation **1** Larson BL, Smith VR (eds.) Academic Press, New York, USA, 143

Lohuis JA, Verheijden JH, Burvenich C, van Miert AS 1988 Pathophysiological effects of endotoxins in ruminants 2: metabolic aspects. The Veterinary quarterly **10** 117-125

Lohuis JA, Kremer W, Schukken YH, Smit JA, Verheijden JH, Brand A, Van Miert AS 1990 Growth of *Escherichia coli* in milk from endotoxin-induced mastitic quarters and the course of subsequent experimental *Escherichia coli* mastitis in the cow. Journal of dairy science **73** 1508-1514

Lough DS, Beede DL, Wilcox CJ 1990 Effects of feed intake and thermal stress on mammary blood flow and other physiological measurements in lactating dairy cows. Journal of dairy science **73** 325-332

Marsál K 1993 Doppler ultrasonography: techniques. In: Hanson A, Spencer JAD, Rodeck CH (eds.): The circulation. Cambridge University Press, England, 296-322

McDowell GH, Hart IC 1984 Responses to infusion of growth hormone into the mammary arteries of lactating sheep. Canadian journal of animal science **64** 306

Menzies FD, Bryson DG, McCallion T, Matthews DI 1995 A study of mortality among suckler and dairy cows in Northern Ireland in 1992. The Veterinary record **137** 531-536

Meurer DG 1999 [Stages of the inflammation]. In: Allgemeine Pathologie: Kompendium für die Veterinärmedizin. Schattauer Verlagsgesellschaft mbH, Stuttgart, Germany, 118-119

Miller GY, Bartlett PC, Lance SE, Anderson J, Heider LE 1993 Costs of clinical mastitis and mastitis prevention in dairy herds. Journal of the American Veterinary Medical Association **A 202** 1230-1236

Mottram T, Rudnitskaya A, Legin A, Fitzpatrick JL, Eckersall D 2007 Evaluation of a novel chemical sensor system to detect clinical mastitis in bovine milk. Biosensors & bioelectronics **22** 2689-2693

Nickerson SC, Boddie RL, Owens WE, Watts JL 1990 Effects of novel intramammary device models on incidence of mastitis after experimental challenge. Journal of dairy science **73** 2774-2784

Nielsen MO, Jakobsen K, Jørgensen JN 1990 Changes in mammary blood flow during the lactation period in goats measured by the ultrasound Doppler principle. Comparative biochemistry and physiology **97A** 519-524

Nourshargh S, Perkins JA, Showell HJ, Matsushima K, Williams TJ, Collins PD 1992 A comparative study of the neutrophil stimulatory activity in vitro and pro-inflammatory properties in vivo of 72 amino acid and 77 amino acid IL-8. Journal of immunology **148** 106-111

Olde Riekerink RG, Barkema HW, Kelton DF, Scholl DT 2008 Incidence rate of clinical mastitis on Canadian dairy farms. Journal of dairy science **91** 1366-1377

Opdebeeck JP, Frost AJ, O´Boyle D 1988 Adhesion of *Staphylococcus aureus* and *Escherichia coli* to bovine udder epithelial cells. Veterinary microbiology **16** 77-86

O´Reilly KM, Green MJ, Peeler EJ, Fitzpatrick JL, Green LE 2006 Investigation of risk factors for clinical mastitis in British dairy herds with bulk milk somatic cell counts less than 150,000 cells/ml . The Veterinary record **158** 649-653

Paape MJ, Mehrzad J, Zhao X, Detilleux J, Burvenich C 2002 Defense of the bovine mammary gland by polymorphnuclear neutrophil leukocytes. Journal of mammary gland biology and neoplasia **7** 109-121

Park YH, Joo YS, Park JY, Moon JS, Kim SH, Kwon NH, Ahn JS, Davis WC, Davies CJ 2004 Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows. Journal of veterinary science **5** 29-39

Peaker M 1980 The effect of raised intramammary pressure on mammary function in the goat in relation to cessation of lactation. Journal de physiologie **301** 415

Peeler EJ, Otte MJ, Esslemont RJ 1994 Inter-relationships of periparturient diseases in dairy cows. The Veterinary record **134** 129–132

Peeters G, Coussens R, Sierens G 1949 Physiology of the nerves in the bovine mammary gland. Archives internationales de pharmacodynamie et de thérapie **79** 75

Peeters G, Bouckaert JH, Oyaert W 1952 The influence of unilateral lumbar sympathectomy on the udder of the sheep. Archives internationales de pharmacodynamie et de thérapie **89** 197

Pensel J, Warnking R 1993 [Instrumental technology]. In: Sohn C, Stolz W, Bastirt G (eds.): Dopplerultrasonographie in der Gynäkologie und Geburtshilfe. Verlag Georg Thieme, Stuttgart, Germany 11-15

Petzl W, Zerbe H, Günther J, Yang W, Seyfert H-M, Nürnberg G, Schuberth H-J 2008 *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Veterinary research **39** 18

Piccione G, Arcigli A, Fazio F, Giudice E, Caola G 2004a Pulsed wave-doppler ultrasonographic evaluation of mammary blood flow speed in cows during different productive periods. Acta Scientiae Veterinariae **32** 171-175

Piccione G, Arcigli A, Assenza A, Percipalle M, Caola G 2004b Pulsed Wave-Doppler Ultrasonographic Evaluation of the Mammary Blood Flow in the Ewe. Acta veterinaria **73** 23-27

Pourcelot L 1974 [Clinical applications of trancutaneous Doppler examinations]. In: Peronneau P (eds.): Velocimetric ultrasonore Doppler. Inserm 34, 7-11 Octobre, Paris, France 213-240

Prakash BS, Meyer HHD, Schallenberger E, van de Wiel DFM 1987 Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the antibody technique. Journal of steroid biochemistry **28** 623-627

Prosser CG, Fleet IR, Corps AN, Froesch ER, Heap RB 1990 Increase in milk secretion and mammary blood flow by intra-arterial infusion of insulin-like growth factor-I into the mammary gland of the goat. The Journal of endocrinology **126** 437

Prosser CG, Davis SR, Farr VC, Moore LG, Gluckman PD 1994 Effects of close arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats. The Journal of endocrinology **142** 93

Prosser CG, Davis SR, Farr VC, Lacasse P 1996 Regulation of blood flow in the mammary microvasculature. Journal of dairy science **79** 1184-1197

Pyörälä SHK, Pyörälä EO 1998 Efficacy and parenteral administration of three antimicrobial agents in treatment of clinical mastitis in lactating cows: 487 cases (1989-1995). Journal of the American Veterinary Medical Association **212** 407-412

Pyörälä S 2003 Indicators of inflammation in the diagnosis of mastitis. Veterinary research **34** 565-578

Rajala-Schultz PJ, Gröhn YT 1999 Culling of dairy cows. Part I. Effects of diseases on culling in Finnish Ayrshire cows. Preventive veterinary medicine **41** 195-208

Rasmussen F 1965 The mammary blood flow in the cow as measured by antipyrine absorption method. Acta veterinaria Scandinavica **6** 135

Raubertas RF, Shook GE 1982 Relationship between lactation measures of somatic cell concentration and milk yield. Journal of dairy science **65** 419-425

Rauch A, Krueger L, Miyamoto A, Bollwein H 2008 Colour Doppler sonography of cystic ovarian follicles in cows. The Journal of reproduction and development **54, 6** 447-453

Reynolds M, Linzell JL, Rasmussen F 1968 Comparison of four methods for measuring mammary blood flow in conscious goats. The American journal of physiology **274** 1415

Schalm OW, Noorlander DO 1957 Experiments and observations leading to development of the California mastitis test. Journal of the American Veterinary Medical Association **130** 199-204

Schmauder S 2003 [Cyclic and inflammatory changes in the endometrial echostructure of cows with consideration to the expression of nitric oxide synthase]. Ludwig-Maximilians-University Munich, Germany diss

Schmucker N, Schatzmann U, Budde K, Gundel M, Jäggin CE, Meier H 2000 Duplex-ultrasonographic evaluation of the common carotid artery in the resting, sedated and anesthetized horse. Veterinary radiology & ultrasound **41** 168-171

Schukken YH, Grommers FJ, van de Geer D, Erb HN, Brand A 1991. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 2. Risk factors for *Escherichia coli* and *Staphylococcus aureus.* Journal of dairy science **74** 826–832

Schukken YH, Leslie KE, Barnum DA, Mallard BA, Lumsden JH, Dick PC, Vessie GH, Kehrli ME 1999 Experimental Staphylococcus aureus intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. Journal of dairy science **82** 2393-2401

Seegers H, Fourichon C, Beaudeau F 2003 Production effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary research **34** 475-491

Shuster DE, Lee EK, Kehrli ME 1996 Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. American journal of veterinary research **14** 1569-1575

Smith KL, Todhunter DA, Schoenberger PS 1985 Environmental pathogens and intramammary infection during the dry period. Journal of dairy science **68** 402-417

Stuart B, Drumm J, Fitzgerald DE, Duignan NM 1980 Fetal blood velocity waveforms in normal pregnancy. British journal of obstetrics and gynaecology **87** 780-785

Suriyasathaporn W, Schukken YH, Nielen M, Brand A 2000 Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. Journal of dairy science **83** 1248-1255

Svennersten-Sjaunja K, Olsson K 2005 Endocrinology of milk production. Domestic animal endocrinology **29** 241-258

Tekay A, Martikainen H, Jouppila P 1996 Comparison of uterine blood flow characteristics between spontaneous and stimulated cycles before embryo transfer. Human reproduction **11** 364-368

Thivierge MC, Petitclerc D, Bernier JF, Couture Y, Lapierre H 2000 External pudic venous reflux into the mammary vein in lactating dairy cows. Journal of dairy science **83** 2230-2238

Vandeputte-Van Messom G, Burvenich C, Roets E, Massart-Leën AM, Heyneman R, Kremer WD, Brand A 1993 Classification of newly calved cows into moderate and severe responders to experimentally induced Escherichia coli mastitis. Journal of dairy research **60** 19-29

Vangroenweghe F, Duchateau L, Burvenich C 2004a. Moderate inflammatory reaction during experimental *Escherichia coli* mastitis in primiparous cows. Journal of dairy science **87** 886–895

Vangroenweghe F, Rainard P, Paape MJ, Duchateau L, Burvenich C 2004b. Increase of Escherichia coli inoculum doses induces faster innate immune response in primiparous cows. Journal of dairy science. **87** 4132–4144

Vanselow J, Yang W, Herrmann J, Zerbe H, Schuberth HJ, Petzl W, Tomek W, Seyfert HM 2006 DNA-remethylation around a STAT5-binding enhancer in the alphaS1-casein promoter is associated with abrupt shutdown of alphaS1-casein synthesis during acute mastitis. Journal of molecular endocrinology **37** 463-477

Van Werven T, Noordhuizen-Stassen EN, Daemen AJJM, Schukken YH, Brand A, Burvenich C 1997 Preinfection in vitro chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. Journal of dairy science **80** 67-74

Viguier C, Arora S, Gilmartin N, Welbeck K, O´Kennedy R 2009 Mastitis detection: current trends and future perspectives. Trends in biotechnology **27** 486-493

Waite LR, Ford SP, Young DF, Conley AJ 1990 Use of ultrasonic Doppler waveforms to estimate changes in uterine artery blood flow and vessel compliance. Journal of animal science **68** 2450-2458

Ward W R, Hughes JW, Faull WB, Cripps PJ, Sutherland JP, Sutherst JE 2002 Observational study of temperature, moisture, pH and bacteria in straw bedding, and faecal consistency, cleanliness and mastitis in cows in four dairy herds. The veterinary record **151** 199–206

Wegner TN, Schuh JD, Nelson FE, Stott GH 1976 Effect of stress on blood leucocyte and milk somatic cell counts in dairy cows. Journal of dairy science **59** 949-956

Wenz JR, Barrington GM, Garry FB, Ellis RP, Magnuson RJ 2006 *Escherichia coli* isolates´ serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. Journal of dairy science **89** 3408-3412

Zadoks RN, Allore HG, Barkema HW, Sampimon OC, Wellenberg GJ, Grohn YT, Schukken YH 2001 Cow- and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. Journal of dairy science **84** 2649–2663

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